PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B2

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/38, 31/415 — A1

(11) International Publication Number: WO 97/06819

(43) International Publication Date: 27 February 1997 (27.02.97)

(21) International Application Number: PCT/US96/13258

(22) International Filing Date: 15 August 1996 (15.08.96)

(30) Priority Data:
60/002,362 15 August 1995 (15.08.95) US

(71) Applicant: ALTEON INC. [US/US]; 170 Williams Drive, Ramsey, NJ 07446 (US).

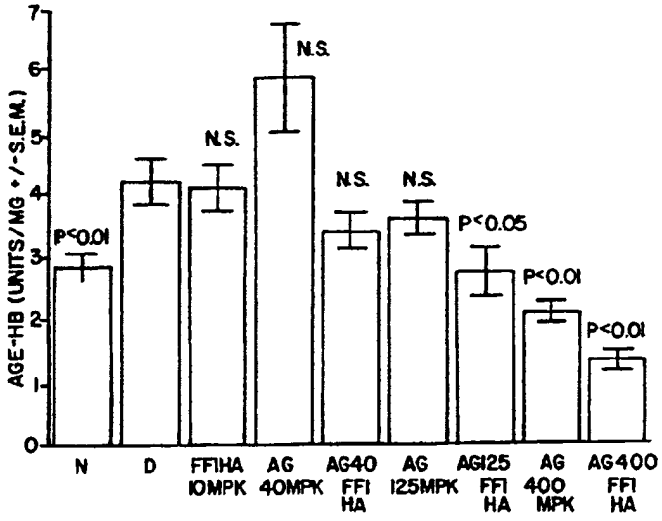
(72) Inventor: MALLON, Veronica; 395 North Little Tor Road, New City, NY 10965 (US).

(74) Agents: FEHLNER, Paul, F. et al.; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COMPOSITIONS AND METHODS FOR ELMINATION OF ADVANCED GLYCOSYLATION ENDPRODUCTS (IN VIVO)



(57) Abstract

The present invention relates to methods and agents for treating diseases or disorders associated with accumulation of advanced glycosylation endproducts (AGEs). More particularly, the invention is directed to methods for removing accumulated AGEs from tissues *in vivo*, and to inhibition of adverse sequelae that may occur with the removal of accumulated AGEs. In specific examples, co-administration of 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI) conjugated to human albumin or hexanoic acid and aminoguanidine is shown to greatly reduce the level of immunoglobulin trapping in kidney vasculature, increase tail collagen solubility, and decrease levels of AGEs in the aorta compared to either FFI or aminoguanidine alone. In a specific aspect, FFI may be covalently coupled to aminoguanidine. In another embodiment, a colony stimulating factor, in particular GM-CSF, or an inflammatory cytokine, in particular MIF or MIP-1β, can be administered to increase recognition and uptake of AGEs.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

COMPOSITIONS AND METHODS FOR ELIMINATION OF ADVANCED GLYCOSYLATION ENDPRODUCTS *IN VIVO*

FIELD OF THE INVENTION

The present invention relates to methods and agents for treating diseases or disorders associated with accumulation of advanced glycosylation endproducts (AGEs). More particularly, the invention is directed to methods for removing accumulated AGEs from tissues *in vivo*, and to reverse the biochemical alterations associated with AGEs.

BACKGROUND OF THE INVENTION

The reaction between glucose and proteins has been known for some time, and in its earliest manifestation, was identified in the appearance of brown pigments during the cooking of food. This phenomenon was identified by Maillard in 1912, who observed that glucose and other reducing sugars react with amino acids to form adducts that undergo a series of dehydrations and rearrangements to form stable brown pigments. Maillard L.C. (1912) *C.R. Acad. Sci.*, Vol. 154, pp. 66-68.

This phenomenon was found in recent years to have a parallel *in vivo*. Accordingly, the nonenzymatic reaction between glucose and the free amino groups on proteins to form a stable amino 1-deoxy ketosyl adduct, known as the Amadori product, has been shown to occur with hemoglobin, wherein a rearrangement of the Amadori product formed at the amino terminal of the beta chain of hemoglobin by reaction with glucose, forms the adduct known as hemoglobin A_{1c}. This initial reaction of the Maillard sequence was also found to occur with a variety of other body proteins, such as lens crystallins, collagen and nerve proteins. See Bunn, H.F., Haney, D.N., Gabbay, K.H. and Gallop, P.H., (1975) *Biochem. Biophys. Res. Comm.*, Vol. 67, pp. 103-109; Koenig, R.J., Blobstein, S.H. and Cerami, A., (1977) *J. Biol. Chem.*, Vol. 252, pp. 2992-2997; Monnier, V.M. and Cerami, A., (1983) MAILLARD REACTION IN FOOD AND NUTRITION, ed. Waller, G.A., *American Chemical Society*, Vol. 215, pp. 431-448; and Monnier, V.M. and Cerami, A., (1982) *Clinics in Endocrinology and Metabolism*, Vol. 11, pp. 431-452.

Moreover, brown pigments with spectral and fluorescent properties similar to those of late-stag Maillard products have also been observed *in vivo* in association with several

long-lived proteins, such as lens proteins and collagen from aged individuals. An age-related linear increase in pigment was observed in human dura collagen between the ages of 20 and 90 years. See Monnier, V.M. and Cerami, A., (1981) *SCIENCE*. Vol. 211, pp. 491-493; Monnier, V.M. and Cerami, A., (1983) *BIOCHEM. BIOPHYS. ACTA.*, Vol. 760, pp. 97-103; and Monnier, V.M., Kohn, R.R. and Cerami, A., "Accelerated Age-related Browning of Human Collagen in Diabetes Mellitus", (1984) *PROC. NAT. ACAD. SCI.*, Vol. 81, pp. 583-587. Interestingly, the aging of collagen can be mimicked *in vitro* by the cross-linking induced by glucose; and the capture of other proteins in the formation of adducts by collagen, also noted, is theorized to occur by a cross-linking reaction, and is believed to account for the observed accumulation of albumin and antibodies in kidney basement membrane and cholesterol-bearing low density lipoprotein in the arterial wall. See, Brownlee, M., Pongor, S. and Cerami, A., (1983) *J. EXP. MED.*, Vol. 158, pp. 1739-1744; and Kohn, R.R., Cerami, A. and Monnier, V.M., (1984) *DIABETES*, Vol. 33, No. 1, pp. 57-59. Cerami, A., Vlassara, H., and Brownlee, M., (1985) *METABOLISM*, Vol. 34, pp. 37-44.

A fluorescent chromophore has been isolated and identified which was found to be present in certain browned polypeptides such as bovine serum albumin and poly-L-lysine, and was assigned a structure 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole (hereinafter "FFI"). The compound was found to exist in a tautomeric state and has incorporated in its structure two peptide-derived amine nitrogens. The incorporation of these amine nitrogens and two glucose residues in the compound suggested that its peptide-bound precursor may be implicated in the *in vivo* cross-linking of proteins by glucose which is observed in the late stage of the Maillard process. See, Chang, J.C.F., Ulrich, P.D., Bucala, R., and Cerami, A., (1985) *J. Biol. Chem.*, Vol 260, pp. 7970-7974. This chromophore made possible the identification of the advanced glycosylation endproducts and assisted additional investigations seeking to clarify the protein aging process and if possible, to identify the specific chemistry involved to assist efforts to develop methods and agents for its inhibition. These efforts resulted in the identification of certain compounds that demonstrate the ability to inhibit advanced glycosylation. Primary among these compounds is aminoguanidine.

However, aminoguanidine and other inhibitors of AGE formation have demonstrated no potential to eliminate, or aid in the elimination of, already formed AGEs *in vivo*.

Further work since the development of the inhibitors mentioned above has resulted in the identification of what appears to be an endogenous means for the *in vivo* elimination or removal of advanced glycosylation endproducts. In U.S. Patent No. 4,900,747 to Vlassara et al., issued February 13, 1990, which is specifically incorporated herein by reference in its entirety, a method and associated agents for the inhibition and treatment of protein aging in animals by stimulating the endogenous AGE clearance mechanisms of such animals to increase their recognition of and affinity for AGEs was disclosed. In particular, phagocytic cells such as monocytes and macrophages were treated with an agent capable of causing the phagocytic cell to increase their AGE-specific phagocytic activity. In particular, the inventors reported that AGEs produced naturally or synthetically, alone or bound to a carrier, could effect this AGE uptake pathway *in vivo*. This patent exemplified use of FFI conjugated to HSA as a stimulator of AGE phagocytosis. Clearance of AGEs from tissues by increasing binding, uptake and degradation by phagocytic cells is critical to reversing the adverse symptomatology associated with AGE formation *in vivo*.

However, the present inventors have perceived that the removal of AGEs via phagocytosis, *e.g.*, by stimulating the scavenging functions of the macrophage, may result in a concomitant increase in AGE peptides, secreted by the phagocytic cells, circulating through the kidney vasculature. This in turn could increase the risk of causing the trapping of other proteins in the kidney vasculature, most notably immunoglobulins, which could lead to reduction of kidney function, *e.g.*, from inflammatory reactions. Prior to the work described herein, there was no awareness that formation of AGE peptides following stimulation of the endogenous AGE clearance mechanisms could constitute an adverse sequelae, much less any suggestion of the means to prevent such adverse sequelae.

Accordingly, the present invention provides an advance over the prior art by providing for clearance of AGEs while avoiding complications.

The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method and associated agents are disclosed for the inhibition and treatment of protein aging in animals by stimulating the bodies of such animals to increase their recognition of and affinity for advanced glycosylation endproducts, while preventing or minimizing complications that may result from such activities. In particular, phagocytic cells such as monocytes and macrophages are treated with an agent capable of causing the phagocytic cells to increase their activity of recognizing and removing AGE-modified macromolecules such as AGE-proteins, AGE-lipids, and AGE-DNA. The animal is concurrently treated with a compound capable of inhibiting the formation of advanced glycosylation endproducts and, particularly, of inhibiting the cross-linking of AGE-peptides to the renal vasculature.

Thus, in a method for averting the adverse sequelae of the accumulation of advance glycosylation endproducts in the body of an animal, comprising introducing into the body an effective amount of an agent capable of causing the body to increase its activity of recognizing and removing macromolecules that have undergone advance glycosylation, the present invention provides an improvement comprising administering a therapeutically effective amount of a compound capable of inhibiting the formation of advanced glycosylation endproducts by reacting with an active carbonyl moiety on an early glycosylation product and inhibits formation of AGE-mediated crosslinks.

The present invention thus provides for administration of one or more stimulator agents, such as a natural or synthetic advanced glycosylation endproduct alone or bound to a carrier, said carrier including a material selected from carbohydrates, proteins, synthetic polypeptides, lipids, bio-compatible natural and synthetic resins, antigens, and mixtures thereof. The stimulator agents could include other advanced glycosylation endproducts that may be prepared from the reaction between sugars and other macromolecules, and cytokines, chemokines or colony stimulating factors which stimulate phagocytic cells to increase their activity toward advanced glycosylation endproducts. The stimulator agents could also include small synthetic organic molecules which act as AGE receptor modulators.

Accordingly, the stimulator agent may comprise the compound FFI, either alone or bound to a protein such as albumin. Preferably, a homogenetic carrier protein, *i.e.*, a protein native to the animal subject undergoing treatment, is selected. For example, for administration to a human, a preferred carrier protein is human albumin (HA).

Alternately, the stimulator agent may comprise an advanced glycosylation endproduct which is prepared, for example, by the reaction of glucose or glucose -6-phosphate with albumin. This reaction product can be used alone or on a carrier in the same fashion as a FFI-albumin complex.

In a specific embodiment, a colony stimulating factor is administered to stimulate recognition and binding of AGEs. In a specific embodiment, examplified *infra*, the colony stimulating factor (CSF) is granulocyte-macrophage (GM) CSF.

In another embodiment, a cytokine or chemokine that stimulates AGE binding activity is MIF or MIP-1B.

In still another embodiment, a cytokine that also stimulates the activity of recognition and binding of AGEs is the protein known as Tumor Necrosis Factor (TNF) and its variant "cachectin" (see U.S. Patent No. 4,900,747). This material may be administered alone or in conjunction with other stimulator agents.

In addition, the stimulator agents of the present invention may be administered in conjunction with materials identified hereinafter as "co-stimulatory agents". The coadministration of the stimulator agent with the co-stimulatory agents has been found to potentiate the activity of the former. Suitable co-stimulatory agents include inflammatory cytokines or chemokines such as interleukin-1 (IL-1) and gamma-interferon (γ-IFN). The stimulatory cytokines, chemokines, or colony stimulating factors described above can also be used as co-stimulatory agents, *e.g.*, in combination with a stimulatory AGE such as FFI.

The inhibitory compounds of the invention are capable of inhibiting, and in some embodiments, reversing the formation of advanced glycosylation endproducts. The rationale of the invention is to use agents that block the post-glycosylation step, *i.e.*, the

formation of fluorescent chromophores and/or molecular cross-links whose presence is associated with, and leads to, the adverse sequelae of glycosylation, in combination with agents that induce removal of AGE-modified molecules from tissues. Generally, an inhibitor of the formation of AGEs includes compounds that react with a carbonyl moiety of an early glycosylation product. Representative of such advanced glycosylation inhibitors are aminoguanidine, lysine, and α-hydrazinohistidine. In a specific embodiment, the inhibitor is aminoguanidine (AG) and derivatives thereof. Pharmaceutical compositions and methods involving AG and derivatives thereof are well known.

In another embodiment, compositions and methods for inhibiting and reversing nonenzymatic cross-linking (protein aging) as described in International Patent Publication WO 96/22095 by Cerami et al (July 25, 1996). Accordingly, compositions and methods are disclosed which utilize an agent capable of inhibiting the formation of advanced glycosylation endproducts of target proteins, and which additionally reverse pre-formed crosslinks in the advanced glycosylation endproducts by cleaving alpha-dicarbonyl-based protein crosslinks present in the advanced glycosylation endproducts. Certain agents useful are thiazolium salts. The method comprises contacting the target protein with the composition.

In a specific embodiment, an stimulatory agent can be chemically coupled to an inhibitory compound to create a single molecule capable both of stimulating the uptake of AGE-modified molecules and inhibiting reactivity of AGEs *in vivo*. Preferably the stimulatory agent is an AGE. In a more preferred embodiment, the stimulatory agent is FFI, and the inhibitor is aminoguanidine. The two can be coupled using standard cross-linking technology, such as carbodiimide-mediated condensation or coupling via a bifunctional linker.

A further alternative embodiment of the method of the present invention and one which may be practiced independently or conjointly with the above recited method, is the *ex vivo* treatment of the phagocytic cells to expose them to the stimulator agents. For example, a patient may be given an extracorporeal blood treatment in which blood is diverted out of the body from the arterial and venous system and is directed through a device that contains stimulator agents and/or co-stimulatory agents positioned to come in contact with

the phagocytic cells within the blood. The stimulator agents and/or co-stimulatory agents may be immobilized or may be allowed to enter the flow of the blood. In this embodiment, the compound capable of inhibiting formation of AGEs is added to the blood returned to the patient, or administered separately to the patient.

In the instance where the method comprises the *in vivo* administration of the stimulator agent and/or stimulatory agents, administration may be accomplished by known techniques, including transmucosal techniques, such as nasal administration, a bucal patch, sublingual, and rectal or vaginal (via a suppository); oral; and parenteral techniques such as intradermal, subcutaneous, subdermal, intramuscular, intravenous, intraarteriole, intraventricular, or intraperitoneal injection, catheterization or other conventional means. The stimulator agents and/or inhibitor compounds, or mixtures of them, may be prepared in suitable pharmaceutical compositions for such administration.

In a further aspect of the present invention, phagocytic cells may be stimulated to increase their ability to recognize and remove target macromolecules by adjustment of the insulin level in the body fluid. In particular, artificial reduction of insulin levels may be achieved by dietary manipulation and/or by the use of pancreatic beta-cell suppression, conducted alone or in combination with the administration of the agents discussed above. This additional method exerts a positive effect on the activity of the phagocytic cells and promotes the increased uptake and elimination of advanced glycosylation endproducts in accordance with the present invention. In this embodiment, manipulation of insulin levels to stimulate phagocytic cell uptake of AGE-modified molecules is accompanied by administration of an inhibitor of AGE formation.

In addition, the present invention relates to therapeutic methods for the treatment of the various adverse sequelae of the build-up of advanced glycosylation endproducts in the body. In particular, pathologies such as age related or diabetes related hardening of the arteries, skin wrinkling, arterial blockage, and diabetic retinal and renal damage, which are all the result of the excessive build-up or trapping that occurs as the presence of advanced glycosylation endproducts increases, can be treated. Accordingly, a therapeutic method in accordance with the present invention generally seeking to avert such pathologies contemplates the coordinated administration of the agents of the present invention either directly or in suitable pharmaceutical compositions to stimulate the

phagocytic cells to remove advanced glycosylation endproducts from the body with greater speed and efficiency, concurrently with administration of a compound capable of inhibiting the formation of AGEs, to thereby more completely avert the onset of the pathologies recited herein. Specific administrative protocols may vary and would be determined upon the specific instruction of qualified medical or veterinary practitioners.

The present method has particular therapeutic application as the Maillard process acutely affects several of the significant protein masses in the body, among them collagen, elastin, lens proteins, and the kidney glomerular basement membranes. These proteins deteriorate both with age (hence the application of the term "protein aging") and as a result of prolonged exposure to blood sugar and AGE formation. Consequently, the enhanced ability to remove glycosylation endproducts from the animal's system while avoiding further reactivity of the products of the removal process carries the promise of favorably treating the significant adverse effects of numerous pathologies including diabetes, with concomitant improvement in the quality and perhaps duration of patient life.

Accordingly, it is a principal object of the present invention to provide a method for improved sequestration and removal of advanced glycosylation endproducts from animal systems.

It is a further object of the present invention to provide a method as aforesaid which is characterized by the stimulation of phagocytic cells to increase their affinity and capability for the binding, uptake and degradation of advanced glycosylation endproducts, while preventing reaction of the AGE-metabolic products of the uptake and degradation processes with proteins in the vasculature.

It is a yet further object of the present invention to provide agents capable of stimulating phagocytic cells to bind, take up and degrade advanced glycosylation endproducts in the method as aforesaid, while concurrently providing compounds capable of inhibiting the formation of advanced glycosylation endproducts.

It is a still further object of the present invention to provide therapeutic methods for treating the adverse consequences of protein aging.

Other objects and advantages will become apparent to those skilled in the art from a consideration of the ensuing description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Results of rat aortic AGE analysis. Rat aortas from control (N) and subject rats (diabetic control -- D, diabetic receiving aminoguanidine -- AG, diabetic receiving FFI-HA -- FFI-HA, and diabetic receiving aminoguanidine and FFI-HA -- FFI/AG) were obtained and analyzed for AGE level as described in the Materials and Methods section of Example 1. An asterisk indicates $p < 0.05$ compared to the diabetic control rats; N.S. indicates no significance in the observed difference compared to diabetic control rats.

FIGURE 2. Results of tail tendon collagen AGE assay. Rat tails from control (N-O) and diabetic (D-O) rats, and diabetic rats receiving aminoguanidine (A), FFI-HA (B), or aminoguanidine and FFI-HA (C) were obtained and analyzed for AGE content as described in the Materials and Methods section for Example 2. ***, $p < 0.001$ compared to diabetic control rats; *, $p < 0.05$ compared to diabetic control rats.

FIGURE 3. Results of IgG trapping assay. Glomerular basement membranes were isolated from frozen kidneys and evaluated for the presence of rat IgG. Samples were tested from normal control (N-O) rats, diabetic control rats (D-O), diabetic rats receiving aminoguanidine (A), diabetic rats receiving FFI-HA (B), and diabetic rats receiving aminoguanidine and FFI-HA (C) as described in the materials and methods for Example 3. *, $p < .05$; N.S. indicates no significant difference from the value for rat diabetic controls.

FIGURE 4. Summary of urinary AGE data. Urinary AGE output from 24 hour urines was measured by ELISA as described in the Examples, *infra*. N-O, normal control rats; D-O, diabetic control rats; A, diabetic rats receiving aminoguanidine; B, diabetic rats receiving FFI-HA; C, diabetic rats receiving aminoguanidine and FFI-HA.

FIGURE 5. Hemoglobin-AGE levels in diabetic rats. Hemoglobin-AGE levels were determined by an ELISA assay. FFI was administered at 10 mg/kg/day, and aminoguanidine was administered at three dosages: 40, 125, and 400 mg/kg/day. N=normal; D=diabetic control (untreated); FFIHA=FFI-hexanoic acid conjugate; AG=aminoguanidine. N.S.=no significance.

FIGURE 6. Effect of MIF on AGE receptor expression. RAW 264.7 cells were incubated for one or three days with 100, 10, or 1 ng/ml MIF. The cells were washed three times to remove MIF and AGE receptor expression measured in a radioligand binding assay.

FIGURE 7. Effect of MIP-1ß on AGE receptor expression. RAW 264.7 cells were incubated with 1, 0.1, 0.01, and 0.004 μg/ml MIP-1ß and assayed as described in Figure 6.

FIGURE 8. Effect of GM-CSF on AGE receptor expression. RAW 264.7 cells were incubated with 100 and 10 ng/ml of GM-CSF as described in Figures 6 and 7. Lack of AGE receptor expression at the higher dose of GM-CSF after three days is probably the result of a feedback mechanism.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, a composition and associated methods have been developed for enhancing the removal of advanced glycosylation endproducts in animals, to treat protein aging and thereby inhibit the adverse effects thereof. Specifically, phagocytic cells such as monocytes and macrophage cells are exposed to one or more stimulator agents which enhance the ability of such phagocytic cells to recognize, bind, and degrade advanced glycosylation endproducts, while at the same time, or shortly thereafter, the animal is treated with an effective amount and regimen of a compound that is capable of inhibiting the formation of AGEs and AGE-protein cross-linking.

The present invention is predicated upon the observation that monocytes and macrophage cells have the ability to recognize, remove and degrade macromolecules that have undergone glucose-mediated damage and thus have undergone advanced glycosylation

endproduct formation, particularly as set forth in U.S. Patent No. 4,900,747 to Vlassara et al., issued February 13, 1990, which is specifically incorporated herein by reference in its entirety. In particular, it has been determined that monocytes and macrophages have a specific surface receptor for the glucose-altered macromolecules that allows the cells to perform their recognition, removal, and degrading functions with respect thereto.

Previous work with respect to glucose-mediated changes in proteins has established that glucose reacts with such macromolecules and leads to changes in their properties that interfere with normal function. Glucose is known to react specifically with the amino groups of proteins without the aid of enzymes, leading them to become cross-linked or to covalently bind to and thereby trap other proteins.

In particular, glucose may initially react with an amino group on a protein and thereby form what is known as a reversible Schiff base adduct. The adduct then rearranges to form a more stable, but still reversible Amadori product. The Amadori product, which is an incipiently glycated protein, may then undergo further reactions, which in one instance, include the reaction with a second glycated amino group of a protein, whereby a cross-link is formed. The result of this reaction is the conversion of the two glucose moieties and their amino groups into an advanced glycosylation endproduct compound.

The structure of a model AGE produced *in vitro* has been identified as FFI. This compound is part of a complex family of advanced glycosylation endproducts that exhibits yellow color and fluoresces. The presence of AGE compounds similar to this compound has been confirmed by the *in vitro* reaction of glucose and proteins, whereby the resulting mass is observed to become yellow to brown in color. These advanced glycosylation endproducts form simply from the reaction of sugars with proteins and other macromolecules, including DNA and lipids. However, there is no direct evidence that FFI is formed *in vivo*.

The term "glycosylation" is used herein to refer to the non-enzymatic reaction of reducing sugars with a nucleophile, in particular an amine group, on a molecule, such as albumin, which leads to formation of AGEs. These processes are well known in the art, as described above. An alternative term for this process that has come more frequently into use is "glycation."

The Amadori product can also react with a second glucose, and the resulting doubly glycated derivative can then react directly with the amino group of a non-glycosylated protein to form an advanced glycosylation endproduct (AGE) and to thereby link the proteins together. This phenomenon has been termed "trapping" and has been demonstrated *in vitro* by the reaction of collagen, a normally insoluble, structural protein, and low-density lipoprotein, which is a circulating, soluble protein, as well as collagen with albumin and IgG. [See Brownlee, et al., *Diabetes*, 34:938-941 (1985); Brownlee, *et al.*, *J. Exp. Med.*, **158**:1739-1744 (1983)].

As used herein, the term "AGE-" can refer to the compound formed by this process, or to the macromolecule which it modifies as the reaction product of either an advanced glycosylation endproduct or a compound which forms AGEs and the compound so modified, such as albumin moiety. AGE-modified molecules can be formed *in vitro* or by reacting a molecule, preferably one that contains an amino group, such as a protein or peptide, with an AGE, for example by conjugating the AGE to the protein with a linker. Alternatively, AGE-modified molecules can be formed *in vitro* or *in vivo* by reacting an amine containing molecule, such as a protein, peptide, nucleic acid, or an amine-containing lipid by with a compound such as a reducing sugar, *e.g.*, glucose, until the molecule is modified to form the AGE-peptide.

Internal cross-linking of proteins or cross-linking of two adjacent proteins may change the mechanical properties of structural proteins. Changes in the immunologic, enzymatic, physical, and other properties of proteins, nucleic acids, and lipids as a result of cross-linking and trapping are also known. Glucose mediated cross-linking and trapping of proteins is a normal process in the body, which over time leads to pathology in many tissues and organs. The rate of glycation, and hence onset of pathologies, is sensitive to the endogenous glucose concentration. For example, it has been observed that the abnormally high levels of glucose in the blood of diabetics leads to an abnormal increase in the formation of cross-links and trapped proteins, and it is postulated that this may be responsible for the increased morbidity and mortality of the disease.

Trapping leads to an abnormal build up of proteins in locations which can lead to pathology. Examples of the pathology that can be caused by glucose-mediated cross-linking and trapping includes the attachment of lipoprotein and other plasma proteins to

the walls of coronary arteries, with the consequent build-up of proteins and cholesterol resulting in coronary arterial blockage and myocardial infarction. Similarly, cross-linking of collagen in the arterial wall can stiffen the mechanical properties of the arterial wall leading to circulatory problems. Trapping and cross-linking of basement membranes of smaller blood vessels in the body and in the kidney leads to peripheral vascular disease and thickening of the kidney basement membrane with subsequent loss of kidney function (nephropathy). Thickening of vessel walls in the brain leads to reduced blood flow and can contribute to the onset of senility; arterial blockage of the brain can cause stroke.

Cross-linking of collagen and other macromolecules in the skin may lead to wrinkling and undesirable changes in skin tone. In addition, AGE-induced protein cross-linking leads to changes in the diabetic eye, particularly damage to the lens and to the vessels of the retina, which latter event leads to a loss of visual acuity and ultimately, to blindness.

Finally, glucose is known to react with DNA, and experiments have shown that AGE-DNA can be mutagenic.

As noted earlier, phagocytic cells are capable of recognizing and removing abnormal macromolecules by means of receptors on their surfaces. Once the abnormal macromolecule is recognized in this way, the phagocytic cell may internalize the macromolecule or particle containing the abnormal macromolecule and may then degrade it. In some instances, the phagocytic cell may in addition secrete enzymes and other factors to help degrade the molecule or particle extracellularly if it cannot be internalized. After the damaged molecule is removed, new growth of normal tissue can ensue, and normal function of the affected area may resume.

Phagocytic cells in the body include numerous types of white blood cells. One type of white blood cell, the monocyte, is produced in the bone marrow, and circulates briefly in the blood and thereafter enters the tissues where it becomes a macrophage. Exposure of the phagocytic cell, either as a monocyte or a macrophage, to certain molecules can regulate the appearance on the surface of the cell of receptors for these molecules.

Thus, the present invention is predicated on the ability of phagocytic cells including monocytes and macrophages to be modified by exposure to certain agents or stimulator

agents that potentiate the capability of these cells with respect to their recognition and affinity for, and capability to degrade advanced glycosylation endproducts (*see* U.S. Patent No. 4,900,747, *supra*), in conjunction with the discovery that this degradation process releases AGE-metabolites, such as AGE peptides, that can lead to cross-linking and capture of proteins in tissues, notably the kidney. In particular, the exposure of these cells to certain stimulator agents has been found to increase the number of receptors developed on these cells and to thereby increase the capacity and efficiency of these cells with respect to the recognition and degradation of advanced glycosylation endproducts, while recognition of the role of concurrent administration of a compound that inhibits formation of AGEs prevents complications that may be associated with the removal process.

Accordingly the method of the present invention generally comprises exposing the animal body to certain stimulator agents, which cause the body, and its phagocytic cells in particular to become activated and to increase its recognition and removal of target macromolecules that have undergone advanced glycosylation, concurrently or followed quickly by administration of a compound that inhibits formation of AGEs, and more particularly, inhibits the formation of AGE-crosslinks. Such an inhibitor of AGE-crosslink formation may also be capable of reversing pre-formed AGE crosslinks.

Stimulator Agents for Inducing Endogenous AGE Removal

The term "stimulator agents" according to the present invention comprises materials such as advanced glycosylation endproducts, either naturally or synthetically formed, which may be employed alone or bound to a carrier. These materials specifically cause the body, *e.g.*, phagocytic cells, to increase the level of recognition and uptake of AGE-modified macromolecules. Examples of stimulator agents are provided in U.S. Patent No. 4,900,747, *supra*.

A preferred stimulator agent is the compound FFI, either alone or bound to a carrier protein such as the protein albumin. The stimulator agent may also comprise an advanced glycosylation endproduct which is prepared, for example, by the reaction of a protein or other macromolecule with a sugar such as glucose, glucose-6-phosphate, or others. This reaction product could be used alone or could be combined with a carrier in the same fashion as the FFI-albumin complex.

The carrier may be selected from the group consisting of carbohydrates, proteins, synthetic polypeptides, lipids, bio-compatible natural and synthetic resins, antigens and mixtures thereof.

The term "stimulator agent" also includes cytokines, chemokines, or colony stimulating factors that stimulate phagocytic cells to increase their activities toward advanced glycosylation endproducts. A preferred colony stimulating factor (CSF) is granulocyte-macrophage CSF. Preferred chemokines are MIF and MIP-1β. A particular cytokine that has been found to function as a stimulator agent is the protein known as Tumor Necrosis Factor (TNF) and its variant named "cachectin". These materials may be administered alone or in conjunction with other stimulator agents.

In addition, the stimulator agents of the present invention may be administered in conjunction with materials identified hereinafter as "co-stimulatory agents". The coadministration of the stimulator agent with the co-stimulatory agents has been found to potentiate the activity of the former. Suitable co-stimulatory agents include cytokines such as interleukin-1 (IL-1) and gamma-interferon (γ-IFN).

In a specific embodiment of preparation of the stimulator agent comprising FFI coupled to a carrier molecule such as albumin, the synthetic compound FFI-hexanoic acid may be used in its preparation. A water-soluble carbodiimide is used to attach the acid moiety of the FFI-hexanoic acid to an amino group on the protein. This conjugate, after purification, is used *in vitro* to stimulate macrophages. After incubation for 4 to 24 hours, it can be shown that such macrophages will more actively bind, internalize, and degrade AGE-albumin (*see* U.S. Patent No. 4,900,747, *supra*).

Alternatively, FFI can be used alone, *i.e.*, unmodified, or bound to a small compound, *e.g.*, a, C_1 to C_{12} hydrocarbon, which may contain one or more heteroatoms or functional groups. For example, as shown in the Examples, *infra*, FFI conjugated to hexanoic is active.

AGE Inhibitory Compounds

The term "inhibitory compound" as used herein refers to a compound capable of inhibiting the formation of advanced glycosylation endproducts. The rationale of the invention is to

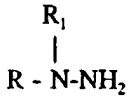
use agents which block the post-glycosylation step, *i.e.*, the formation of fluorescent chromophores and/or molecular cross-links whose presence is associated with, and leads to, the adverse sequelae of glycosylation. An ideal agent would prevent the formation of AGE-associated chromophores and/or cross-links bridging proteins and covalently trapping proteins onto other proteins. In a further embodiment, the inhibitor of AGE formation can reverse pre-formed crosslinks in the AGEs.

The present invention does not attempt to prevent initial protein glycosylation reactions, as it would be nearly impossible to use agents which prevent the reaction of glucose with protein amino groups. The agents that are capable of preventing initial glycosylation are likely to be highly toxic, and since the initial glycosylation comes to equilibrium in about three weeks, there is inadequate time available to achieve this objective. Instead, the ideal agent would prevent or inhibit the long-term, post-glycosylation steps that lead to the formation of the ultimate advanced glycosylation end products.

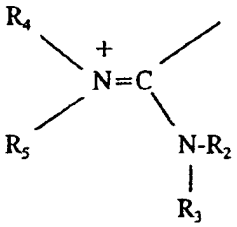
In a further aspect of the invention, an inhibitor of the formation of AGEs includes compounds that react with a carbonyl moiety of an early glycosylation product. Representative of such advanced glycosylation inhibitors are aminoguanidine, lysine, and α-hydrazinohistidine. In a specific embodiment, the inhibitor is aminoguanidine (AG) and derivatives thereof. Pharmaceutical compositions and methods involving AG and derivatives thereof are well known, as described in U.S. Patents No. 4,758,583, issued July 19, 1988; No. 4,908,446, issued March 13, 1990; No. 4,983,604, issued January 8, 1991; No. 5,100,919, issued March 31, 1992; No. 5,106,877, issued April 21, 1992; No. 5,114,943, issued May 19, 1992; No. 5,128,360, issued July 7, 1992; No. 5,130,324, issued July 14, 1992; No. 5,130,337, issued July 14, 1992; No. 5,137,916, issued August 11, 1992; No. 5,140,048, issued August 18, 1992; No. 5,175,192, issued December 29, 1992; No. 5,218,001, issued June 8, 1993; No. 5,221,683, issued June 22, 1993; No. 5,238,963, issued August 24, 1993; No. 5,243,071, issued September 7, 1993; and No. 5,254,593, issued October 19, 1993. Other inhibitors of AGE formation are described in U.S. Applications Serial No. 07/652,575, filed February 8, 1991, now Patent No. 5,258,381; Serial No. 07/889,141, filed May 27, 1992, now Patent No. 5,356,985; Serial No. 07/896,854, filed May 15, 1992, now Patent No. 5,272,176; Serial No. 07/986,661, filed December 8, 1992; Serial No. 07/986,662, filed December 8, 1992, now Patent No. 5,358,960; Serial No. 08/027,086, filed March 5, 1993; and Serial No. 08/095,095, filed

July 20, 1993. Each of the foregoing patents and patent applications is specifically incorporated herein by reference in its entirety. Such inhibitors of AGE formation can be administered directly to the brain or cerebrospinal fluid, *e.g.*, by direct cranial or intraventricular injection, or may pass through the blood brain barrier following administration by parenteral injection, oral administration, skin absorption, etc.

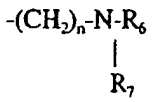
Accordingly, such compounds include a variety of hydrazine derivatives having, for example, a generic formula as follows:



wherein R is a group of the formula



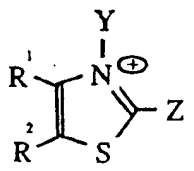
and R_1 is hydrogen or a lower alkyl group of 1-6 carbon atoms, a hydroxyethyl group, or together with R_2 may be a lower alkylene bridge of 2-4 carbon atoms; R_2 is hydrogen or a lower group alkyl of 1-6 carbon atoms or together with R_1 or R_3 is a lower alkylene bridge of 2-4 carbon atoms, amino, hydroxy, or an aminoalkylene group of the formula



wherein n is an integer of 2-7 and R_6 and R_7 are independently a lower alkyl group of 1-6 carbon atoms or together form a part of a cycloalkyl or heterocyclic ring containing from 1 to 2 heteroatoms, of which at least one is nitrogen; and the second of said heteroatoms is selected from the group consisting of nitrogen, oxygen, and sulfur; with the proviso that when the second of said heteroatoms of the heterocyclic ring is nitrogen and forms a piperazine ring; it may be optionally substituted by a substituent that is identical to the portion of the compound on the first nitrogen of the piperazine ring; R_3 is hydrogen, a lower alkyl group of 1-6 carbon atoms, or together with R_2 or R_4 is a lower alkylene bridge of 2-4 carbon atoms; R_4 is hydrogen, a lower alkyl group of 1-6 carbon atoms or together with R_3 is a lower alkylene bridge of 2-4 carbon atoms; or an amino group; R_5 is

hydrogen, or a lower alkyl group of 1-6 carbon atoms; with the proviso that at least one of R_1, R_2, R_3, R_4 or R_5 is other than hydrogen; or R is an acyl or a lower alkylsulfonyl group of up to 10 carbon atoms and R_1 is hydrogen; and their pharmaceutically acceptable acid addition salts.

In a further embodiment, compositions for the inhibition of formation of advanced glycosylation of proteins (protein aging) and for breaking the cross-links that form between advanced glycosylation (glycation) endproducts (AGEs) or between AGEs and other proteins [International Patent Publication WO 96/22095 by Cerami et al. (1995)] as an AGE inhibitory compound of the invention. Such agents inhibit the formation of and reverse the pre-formed advanced glycosylation (glycation) endproducts and break the subsequent cross-links. It is believed that the breaking of the pre-formed advanced glycosylation (glycation) endproducts and cross-links is a result of the cleavage of α dicarbonyl-based protein crosslinks present in the advanced glycosylation endproducts. Certain of the agents useful in the present invention are members of the class of compounds known as thiazoliums. The agents comprise thiazolium compounds having the following structural formula:

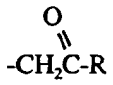


(I)

wherein R^1 and R^2 are independently selected from the group consisting of hydrogen, hydroxy(lower)alkyl, acetoxy(lower)alkyl, lower alkyl, lower alkenyl, or R^1 and R^2 together with their ring carbons may be an aromatic fused ring, optionally substituted by one or more amino, halo or alkylenedioxy groups;

Z is hydrogen or an amino group;

Y is amino, a group of the formula



wherein R is a lower alkyl, alkoxy,

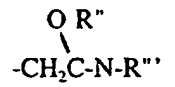
hydroxy, amino or an aryl group, said aryl group optionally substituted by one or more lower alkyl, lower alkoxy, halo, dialkylamino, hydroxy, nitro or alkylenedioxy groups;

a group of the formula

$-CH_2R'$

wherein R' is hydrogen. or a lower alkyl. lower alkynyl, or aryl group;

or a group of the formula



wherein R" is hydrogen and R"' is a lower alkyl group, optionally substituted by an aryl group, or an aryl group, said aryl group optionally substituted by one or more lower alkyl, halo, or alkoxylcarbonyl groups; or R" and R"' are both lower alkyl groups;

X is a halide, tosylate, methanesulfonate or mesitylenesulfonate ion;

and mixtures thereof, and a carrier therefor.

The compounds, and their compositions, utilized in this invention appear to react with an early glycosylation product thereby preventing the same from later forming the advanced glycosylation end products which lead to cross-links, and thereby, to molecular or protein aging and other adverse molecular consequences. Additionally, they react with already formed advanced glycosylation end products to reduce the amount of such products.

Cross-linking Agents

As noted above, in a specific embodiment the present invention contemplates administration of an AGE stimulatory agent chemically coupled to an inhibitory agent to create a single molecule capable both of stimulating the uptake of AGE-modified molecules and inhibiting reactivity of AGEs *in vivo*. The two can be coupled using standard cross-linking technology. In a preferred embodiment, the AGE stimulatory agent is FFI, and the inhibitor is aminoguanidine. In a preferred embodiment, FFI-conjugated to hexanoic acid (which can act as a cross-linking agent) is reacted with aminoguanidine to form the stimulatory agent-inhibitor conjugate. In another embodiment, the ability of AGEs to react with amines can be employed to generate the stimulator-inhibitor conjugate of the invention.

Many strategies are available in the art for conjugating molecules using cross-linkers (also referred to as "linkers" or "spacers"). In general, a molecule, such as the agent of the invention, may be conjugated to another molecule, such as the inhibitor, through a

polyfunctional molecule, *i.e.*, a polyfunctional cross-linker. As used herein, the term "polyfunctional molecule" encompasses molecules having one functional group that can react more than one time in succession, such as formaldehyde (although formaldehyde is not indicated for use due to its potential carcinogenicity), as well as molecules with more than one reactive group. As used herein, the term "reactive group" refers to a functional group on the cross-linker that reacts with a functional group on a molecule (*e.g.*, peptide, protein, carbohydrate, nucleic acid, particularly an AGE or an inhibitor of AGE formation) so as to form a covalent bond between the cross-linker and that molecule. The term "functional group" retains its standard meaning in organic chemistry. The polyfunctional molecules which can be used are preferably biocompatible linkers, *i.e.*, they are noncarcinogenic, nontoxic, and substantially non-immunogenic *in vivo*. Polyfunctional cross-linkers such as those known in the art and described herein can be readily tested in animal models to determine their biocompatibility. The polyfunctional molecule is preferably bifunctional. As used herein, the term "bifunctional molecule" refers to a molecule with two reactive groups. The bifunctional molecule may be heterobifunctional or homobifunctional. A heterobifunctional cross-linker allows for vectorial conjugation. It is particularly preferred for the polyfunctional molecule to be sufficiently soluble in water for the cross-linking reactions to occur in aqueous solutions such as in aqueous solutions buffered at pH 6 to 8, and for the resulting conjugate to remain water soluble for more effective bio-distribution. Typically, the polyfunctional molecule covalently bonds with an amino or a sulfhydryl functional group. However, polyfunctional molecules reactive with other functional groups, such as carboxylic acids or hydroxyl groups, are contemplated in the present invention.

The homobifunctional molecules have at least two reactive functional groups, which are the same. The reactive functional groups on a homobifunctional molecule include, for example, aldehyde groups and active ester groups. Homobifunctional molecules having aldehyde groups include, for example, glutaraldehyde and subaraldehyde. The use of glutaraldehyde as a cross-linking agent was disclosed by Poznansky et al., Science 223, 1304-1306 (1984). Homobifunctional molecules having at least two active ester units include esters of dicarboxylic acids and N-hydroxysuccinimide. Some examples of such N-succinimidyl esters include disuccinimidyl suberate and dithio-bis-(succinimidyl propionate), and their soluble bis-sulfonic acid and bis-sulfonate salts such as their sodium

and potassium salts. These homobifunctional reagents are available from Pierce, Rockford, Illinois.

The heterobifunctional molecules have at least two different reactive groups. The reactive groups react with different functional groups, *e.g.*, present on the agent and the inhibitor. These two different functional groups that react with the reactive group on the heterobifunctional cross-linker are usually an amino group, *e.g.*, the epsilon amino group of lysine; a sulfhydryl group, *e.g.*, the thiol group of cysteine; a carboxylic acid, *e.g.*, the carboxylate on aspartic acid; or a hydroxyl group, *e.g.*, the hydroxyl group on serine. Analogous functional groups can be found on carbohydrates, AGEs, and small molecule inhibitors of AGE formation.

When a reactive group of a heterobifunctional molecule forms a covalent bond with an amino group, the covalent bond will usually be an amido or imido bond. The reactive group that forms a covalent bond with an amino group may, for example, be an activated carboxylate group, a halocarbonyl group, or an ester group. The preferred halocarbonyl group is a chlorocarbonyl group. The ester groups are preferably reactive ester groups such as, for example, an N-hydroxy-succinimide ester group or that of Mal-Sac-HNSA.

According to the invention, the conjugate can be formed between, an amino group on one molecule (or a linker conjugated to the molecule), *e.g.*, the inhibitory compound, and a carboxylic acid group on the other molecule (or linker conjugated to the molecule), *e.g.*, the stimulatory agent. Preferably, such a conjugate is formed through a carbodiimide-mediated condensation reaction. Many carbodiimide reagents are known for use in aqueous, polar, and non-polar solvents, *e.g.*, 1-ethyl-3-dimethylaminopropylcarbodiimide hydrochloride (water soluble), or cyclohexylcarbodiimide (non-polar solvent soluble). Solvents are selected on the basis of relative solubility of the reagents, and may include water, dimethylsulfoxide, dioxane, tetrahydrofuran, ethyl acetate, and mixtures thereof, to mention a few possibilities.

The other functional group typically is either a thiol group, a group capable of being converted into a thiol group, or a group that forms a covalent bond with a thiol group. The covalent bond will usually be a thioether bond or a disulfide. The reactive group that forms a covalent bond with a thiol group may, for example, be a double bond that reacts

with thiol groups or an activated disulfide. A reactive group containing a double bond capable of reacting with a thiol group is the maleimido group, although others, such as acrylonitrile. are also possible. A reactive disulfide group may, for example, be a 2-pyridyldithio group or a 5,5'-dithio-bis-(2-nitrobenzoic acid) group. Some examples of heterobifunctional reagents containing reactive disulfide bonds include N-succinimidyl 3-(2-pyridyl-dithio)propionate (Carlsson, et al., 1978, Biochem J., 173:723-737), sodium S-4-succinimidyloxycarbonyl-alpha-methylbenzylthiosulfate, and 4-succinimidyloxycarbonyl-alpha-methyl-(2-pyridyldithio)toluene. N-succinimidyl 3-(2-pyridyldithio)propionate is preferred. Some examples of heterobifunctional reagents comprising reactive groups having a double bond that reacts with a thiol group include succinimidyl 4-(N-maleimidomethyl)cyclohexahe-1-carboxylate and succinimidyl m-maleimidobenzoate.

Other heterobifunctional molecules include succinimidyl 3-(maleimido)propionate, sulfosuccinimidyl 4-(p-maleimido-phenyl)butyrate, sulfosuccinimidyl 4-(N-maleimidomethyl-cyclohexane)-1-carboxylate, maleimidobenzoyl-N-hydroxy-succinimide ester. The sodium sulfonate salt of succinimidyl m-maleimidobenzoate is preferred. Many of the above-mentioned heterobifunctional reagents and their sulfonate salts are available from Pierce.

Additional information regarding how to make and use these as well as other polyfunctional reagents may be obtained from the following publications or others available in the art:

Carlsson, J. et al., 1978, Biochem. J. 173:723-737.

Cumber, J.A. et al., 1985, Methods in Enzymology 112:207-224.

Jue, R. et al., 1978, Biochem 17:5399-5405.

Sun, T.T. et al., 1974, Biochem. 13:2334-2340.

Blattler, W.A. et al., 1985, Biochem. 24:1517-152.

Liu, F.T. et al., 1979, Biochem. 18:690-697.

Youle, R.J. and Neville, D.M. Jr., 1980, Proc. Natl. Acad. Sci. U.S.A. 77:5483-5486.

Lerner, R.A. et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3403-3407.

Jung, S.M. and Moroi, M., 1983, Biochem. Biophys. Acta 761:162.

Caulfield, M.P. et al., 1984, Biochem. 81:7772-7776.

Staros, J.V., 1982, Biochem. 21:3950-3955.

Yoshitake, S. et al., 1979, Eur. J. Biochem. 101:395-399.

Yoshitake, S. et al., 1982, J. Biochem. 92:1413-1424.

Pilch, P.F. and Czech, M.P., 1979, J. Biol. Chem. 254:3375-3381.

Novick, D. et al., 1987, J. Biol. Chem. 262:8483-8487.

Lomant, A.J. and Fairbanks, G., 1976, J. Mol. Biol. 104:243-261.

Hamada, H. and Tsuruo, T., 1987, Anal. Biochem. 160:483-488.

Hashida, S. et al., 1984, J. Applied Biochem. 6:56-63.

Additionally, methods of cross-linking are reviewed by Means and Feeney, 1990, Bioconjugate Chem. 1:2-12.

Therapeutic Methods and Compositions

Naturally, the present invention includes various therapeutic methods seeking to treat the adverse effects of the buildup of advanced glycosylation endproducts in animals. Such conditions as age- or diabetes- related hardening of the arteries, skin wrinkling, arterial blockage and diabetic retinal and renal damage all result from the excessive buildup or trapping that occurs as advanced glycosylation endproducts increase in quantity. Accordingly, a therapeutic method seeking to avert pathologies caused at least in part by the accumulation of advanced glycosylation endproducts in the body comprises the administration of an agent capable of causing the body to increase its activity of recognizing and removing AGE-modified macromolecules, and a compound capable of inhibiting the formation of advanced glycosylation endproducts. Each may be administered separately or together in suitable pharmaceutical compositions to stimulate the body to increase its activity toward the recognition and removal of such advanced glycosylation endproducts while avoiding complications associated with metabolized AGEs that have been recognized and removed. In particular, the agents are administered to stimulate the phagocytic cells in the body to increase their activity toward the recognition and removal of advanced glycosylation endproducts so that such removal occurs with greater speed and efficiency, and the compounds are administered to prevent cross-linking or further AGE formation by the products of the recognition and removal of AGEs by phagocytic cells. Specific administrative protocols would vary and would be determined upon the instruction of qualified medical or veterinary practitioners.

Accordingly, the present invention also includes suitable pharmaceutical compositions for use in the therapeutic methods of the invention, comprising an agent capable of causing

the body to increase its activity of recognizing and removing AGE-modified macromolecules, and a compound capable of inhibiting the formation of advanced glycosylation endproducts of the present invention prepared in a suitable pharmaceutically acceptable carrier. Such carriers are known, as described above, and may vary in composition and/or concentration depending upon the manner of administration, i.e. oral, parenteral, etc.

A composition comprising "A" (where "A" is a single protein, DNA molecule, vector, recombinant host cell, etc.) is substantially free of "B" (where "B" comprises one or more contaminating proteins, DNA molecules, vectors, etc.) when at least about 75% by weight of the proteins, DNA, vectors (depending on the category of species to which A and B belong) in the composition is "A". Preferably, "A" comprises at least about 90% by weight of the A+B species in the composition, most preferably at least about 99% by weight. It is also preferred that a composition, which is substantially free of contamination, contain only a single molecular weight species having the activity or characteristic of the species of interest.

In a specific embodiment, the invention provides a combination consisting of a macrophage stimulator conjugated to an AGE formation inhibitor such that both individual activities are attained in the new drug conjugate.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable

pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce, *i.e.*, by about 15 percent, preferably by 50 percent, more preferably by 90 percent, and most preferably prevent, a clinically significant deficit in the activity, function, and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

According to the invention, each component of the therapeutic method of the invention may be introduced separately, according to protocols developed to optimize the effectiveness of each agent. For example, the agent capable of causing the body to increase its activity of recognizing and removing AGE-modified macromolecules can be administered in a bolus dose at the initiation of treatment, while the compound capable of inhibiting the formation of AGEs is administered in multiple doses over the therapeutic period, *e.g.*, by infusion by a pump or multiple injections. Alternatively, both components can be admixed for administration in the same composition. For each separately or both together, administration can be accomplished parenterally; transmucosally, *e.g.*, nasally or rectally; orally; or transdermally. Preferably, administration is parenteral, *e.g.*, via intravenous injection, and also including, but is not limited to, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration.

In another embodiment, one or both of the therapeutic components can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid).

In yet another embodiment, one or both of the therapeutic components can be delivered in a controlled release system. For example, the stimulatory agent or the inhibitory compound, or both, may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed.

Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

The present invention will be better understood from a consideration of the following illustrative examples and data, that confirm the activities of the phagocytic cells and their relationship to the stimulator agents discovered in accordance herewith.

EXAMPLES

The following Examples demonstrate the efficacy of aminoguanidine and FFI-HA in combination therapy for treatment of increased levels of AGE complexes *in vivo*. A diabetic rat model was used to study the effects of aminoguanidine administration in combination with FFI-HA on AGE elimination and AGE-related complications. Five groups of six Harlan Lewis rats each were employed. One group was a normal control group, the remaining four groups were made diabetic by treatment with 65 mg/kg of streptozotocin (STZ) given i.v. Eight months after diabetes induction, rats were then subjected to aminoguanidine (40 mg/kg, orally, FFI-HA (10 mg/kg, I.P.), combination, or no treatment (control), and levels of AGEs sampled by competitive ELISA. Rats were treated for a total of 10 weeks. Table 1 represents the conditions for each animal group.

The rats were group housed and fed certified Purina Rat Chow #5002 and given tap water ad Lib.

At the indicated time of sacrifice, the rats were anesthetized and whole blood collected and divided equally into one heparinized and one non-heparinized tube for collection of plasma

and serum, respectively. Serum was collected following a 30 minute incubation at 37°C to promote clotting, and a 30 minute incubation at 4°C to promote clot retraction. The tubes were centrifuged for 15 minutes at 2500 RPM, and a serum removed and put in a labeled microfuge tube and frozen at -20°C until needed. The red cell pellet was delipidated and prepared for an AGE-hemoglobin assay. The kidneys were perfused with saline prior to removal. They were stored frozen in saline at -70°C until used. The tails were collected and stored dry in screw capped plastic tubes until the tail tendon pepsin solubility could be analyzed. A one to two inch segment of aorta was collected from each animal and stored dry in eppendorf microfuge tubes at 70°C for measurement of AGEs.

TABLE 1

Protocol Design for Study

ANIMAL GROUP	TREATMENT			Number of Rats per Group
	STZ (65mg/kg) I.V.	AG (40 mg/kg/day) oral	FFI-HA (10mg/kg/day) I.P.	
A. Normal	-	-	-	6
B. Diabetic C	+	-	-	6
C. Diabetic	+	+	-	6
D. Diabetic	+	-	+	6
E. Diabetic	+	+	+	6

Rats were made diabetic using 65 mg/kg of STZ given I.V. Eight months later treatment was started with either AG alone given by gavage, FFI-HA alone by I.P. injection, or combination therapy. The rats were treated for a total of 10 weeks.

AGE ELISA

AGE measurements were performed in a competitive ELISA. Ninety-six well microtiter plates (NUNC Immunoplate) were coated with AGE-BSA by adding 0.1 ml of a 10 μg/ml solution prepared in 0.1 M $NaHCO_3$, pH 9.6, and incubating for 2 hours at room temperature (RT) or at 4°C overnight. Wells were washed three times with 0.15 ml of PBS containing 0.05% Tween-20. Wells were then blocked by incubation for one hour at RT with 0.15 ml of PBS containing 2% normal goat serum plus 0.1% BSA. After washing three times with PBS-Tween, 50 μl of competing antigen were added to the wells after a series of dilutions in PBS pH 7.4. This was followed by addition of 50 μl of the

primary antibody (1:1000 dilution of rabbit anti-AGE-RNase; Makita et al., 1992, J. Biol. Chem. 267:5133-38; International Patent Publication No. WO 93/13421 by Bucala, July 8, 1993). Plates were covered and incubated at RT 2 hrs. The plates were then washed three times with PBS-Tween. To each well was added 0.1 ml of alkaline phosphatase conjugated goat anti-rabbit IgG (1:5000 in PBS). The plates were incubated for 1 hr at 37°C. The plates were then washed three times with PBS-Tween, followed by one manual wash with 10 mM DEA, pH 9.0. Afterwards, 0.1 ml of substrate (1 mg/ml PNPP in 0.1 M DEA, pH 9.8) was added to each well and color development was allowed to proceed for approximately 2 hrs (until control wells without competing antigen yielded an OD of about 1.8). The absorbance was read at 410 nm on a Dynatech MR 5000 plate reader. The results were read from a standard curve using AGE-BSA as a competitor and plotted as logit (% B/B0) versus log of the AGE concentration. AGE hemoglobin is expressed as AGE units per mg protein.

All AGE determinations were derived from a standard curve using AGE BSA. The results were plotted as % B/Bo vs. the log of the AGE-BSA concentration. This curve was linearized using a program for LOGIT transformations. The AGE-HB is expressed as U/mg of protein. One unit is defined as the amount of antibody-reactive material that was equivalent to 1μg of the AGE-BSA standard.

EXAMPLE 1: PREVENTION OF AGE ACCUMULATION IN AORTIC TISSUE BY ADMINISTERING FFI-HA AND AMINOGUANIDINE

In this Example, aortic tissue was assayed for AGE levels after treatment of diabetic rats with aminoguanidine, FFI-HA, or both to ascertain whether the combination was more beneficial than administration of either alone. The results were compared to AGE levels found in diabetic control rats, and normal, non-diabetic rats.

Materials and Methods

Diabetic animals were first established by administering STZ and eight months later further treated with either aminoguanidine, FFI-HA, both, or no treatment for 10 weeks, as described earlier. To prepare aortas for the AGE assay, aortas from each group of rats

were removed, cleaned of any excess tissue or fat, and rinsed with CMF-PBS solution. Each aorta was frozen with liquid nitrogen in a stainless steel mortar and pestle and then pulverized. Samples were dried in a speed vac overnight at RT. Approximately 10 mg of each individual tissue was removed and delipidated using 5 ml of a 1:1 mixture of chloroform/methanol overnight on an orbital rocker at 4°C. Tissues were centrifuged at 2600 r.p.m. for 15 min., then washed two times with 5 ml of a chloroform/methanol solution (1:5), followed by one wash with 5 ml of methanol only, and then with 10 ml of distilled water. The tissue was finally washed with 10 ml of CMF-PBS. Samples were next digested with 1.5 ml of a collagenase solution (0.4 mg collagenase Type VII (Sigma)/ml CMF-PBS plus one drop of toluene) and digested at 37°C for 3 days on an orbital rocker. The samples were then centrifuged for ten min. at 5,000 r.p.m. in a microfuge. The supernatant was removed and placed in a 70°C water bath for one hour to neutralize enzyme activity prior to AGE analysis using the AGE ELISA assay described *supra*. Supernatants were also assayed for hydroxyproline following hydrolysis in 6 N HCl to assess the amount of collagen present in the AGE assay.

Results

Figure 1 represents the data obtained from the AGE assay of aortic tissue. Each tissue sample tested was taken from either non-diabetic rats or diabetic-induced rats administered aminoguanidine, FFI-HA, both drugs, or neither drug. The data indicate that in normal rat aorta there exists approximately 8 AGE U/mg collagen (AGE Unit is defined in AGE assay above). In contrast, diabetic rats possess greater levels of AGEs, which reach almost 15 U/mg collagen. Treatment of diabetic rats with aminoguanidine reduced this level somewhat to approximately 13 U/mg collagen, while FFI-HA treatment of diabetic rats further reduced AGE content. The combination of both drugs, however, had the greatest cumulative effect on AGE reduction, thereby reducing levels of AGEs to 10 U/mg collagen, which is very close to the normal range.

Discussion

The present Example demonstrates that in subjects treated with both FFI-HA and aminoguanidine AGE, levels are dramatically reduced from deleterious levels found in untreated diabetic subjects.

The efficacy of the combination therapy described in the invention will become more evident with additional Examples which are described below.

EXAMPLE 2: TAIL TENDON COLLAGEN ASSAY

This Example demonstrates the efficacy of combination treatment for individuals susceptible to increased levels of AGEs comprising aminoguanidine and FFI-HA with the rat tail tendon collagen assay. Following the drug treatment period, the tails were removed and tail tendon collagen (TTC) solubility was assessed following limited pepsin digestion, (50 μg/ml pepsin for 4 hrs. in 0.5 N acetic acid). This assay measures the level of solubility of tail tendon collagen, whereby an increase in solubility of TTC suggests lower levels of AGEs and associated cross-links.

Materials and methods

TTC was obtained from rats maintained as described earlier by first removal and freezing at -70°C upon necropsy. Afterwards, the tails were thawed, tendons isolated, washed, and lyophilized. Ten mg samples in duplicate are digested at 4°C for 4 hours in 50 μg/ml of pepsin prepared in 0.5 N acetic acid. The soluble and insoluble collagen were separated by centrifugation. Hydroxyproline contents were determined on the two fractions after acid hydrolysis. Results were expressed as percent soluble hydroxyproline.

Results

The solubility of tail tendon collagen is shown in Figure 2. In non-diabetic rat subjects the level of solubility of collagen was approximately 60% and ranked high as compared with diabetic subjects. The solubility of collagen decreased substantially in the diabetic rats. Administration of aminoguanidine or FFI-HA tended to increase the solubility above that measured for untreated diabetic subjects. The administration of both drugs further increased the solubility almost two times that measured for diabetic rats without any treatment, indicating a significant ($p < 0.05$) reduction in AGE-modified collagen present.

Discussion

The results of the present Example further demonstrate the efficacy of the present invention for treating individuals suspected of having increased tissue levels of AGEs. It

is clear from the graph shown in Figure 2 that a combination of aminoguanidine and FFI-HA was more effective than either one alone.

EXAMPLE 3: ADMINISTRATION OF AMINOGUANIDINE AND FFI-HA IN COMBINATION REDUCES LEVELS OF PROTEIN ENTRAPMENT IN THE KIDNEY AS A RESULT OF AGE ACCUMULATION

This Example considers the problem of increased AGE deposition in the kidney as a result of FFI-HA administration alone to individuals susceptible or having increased levels of AGEs in the tissues. It has been previously demonstrated that administration of FFI-HA to subjects possessing elevated levels of AGEs causes a decrease in tissue levels of AGE compounds due to the scavenging activity of macrophages (U.S. Patent 4,900,747, issued February 13, 1990 to Vlassara et al.). This, in turn, can result in deposition in the kidney of AGE material removed from the tissues. This consequence could eventually prove detrimental to the normal activity of the kidney as well as to circulating immune proteins that may become entrapped in the kidney. The following Example demonstrates reduction of immunoglobulins trapped in the kidney as a result of FFI-HA administration to subjects possessing increased levels of AGE compounds.

Materials and Methods

To determine the efficacy of the administration of aminoguanidine in combination with FFI-HA to reduce the entrapment of proteins in the kidney, rats were pre-treated as described in Table 1. After the ten week period, rats were killed and kidneys removed. The glomerular basement membrane (GBM) was isolated from frozen kidneys using a sequential sieving method which includes a 1 M NaCl wash step to disrupt any non-covalently bound antibody.

Kidneys were removed from the -70°C freezer and allowed to thaw at room temperature. The kidneys from individual animals were placed in 100 X 20 mm petri dishes containing cold Dubelcco's Phosphate Buffered Saline solution (D-PBS) to which has been added 1 mM EDTA and 1 mM PMSF (phenyl methyl sulfonyl flouride), and were kept on ice at all times. The kidneys were cleaned by removing excess capsules, fat and veins, and the cortex was removed by cutting longitudinally or vertically with a razor blade or a sharp No. 10 scalpel. The medulla and papilla were discarded, and the cortex was passed

through a No. 100 (.150 mm mesh) sieve using the bottom of a clean 50 ml Erlenmeyer flask. The No. 100 sieve was rinsed with PBS and the wash is collected into a cl an beaker. The contents of this beaker were then passed through a number 170 sieve (0.090 mm mesh). The eluate from this sieve was kept in a clean beaker on ice while the glomeruli retained on the mesh were collected by washing 3 to 4 times with PBS. The washed glomeruli were collected in a fresh beaker and kept on ice. The eluate from the 170 sieve was then passed through a No. 270 (0.053 mm mesh) sieve. The remaining glomeruli retained on the mesh were washed with D-PBS and saved as described above. The eluate from sieve No. 270 was discarded. The glomeruli preparations from the 170 and 270 sieves were combined and checked microscopically. The collected glomeruli were poured into a 50 ml polypropylene centrifuge tube and pelleted in a swinging bucket rotor at 1500 rpm for 15 minutes at 4°C. The supernatant was discarded and glomeruli were resuspended in 5 mls of cold 1 M NaCl on ice at 4°C. The samples were mixed and sonicated on ice 8 X 30 seconds with 1 minute intervals between sonications. A low range needle probe tip (50 W maximum) with a Braun - Sonic 2000 U Model sonicator was employed. Samples were examined microscopically to ensure that all glomeruli were disrupted, and poured into individual 15 ml Corex tubes to collect GBMs by high speed centrifugation at 16,000 rpm for 30 minutes at 4°C. The pellet was resuspended with 0.5 mls of deionized water by sonicating in a 15 ml Corex tube for a uniform GBM preparation. The samples were stored frozen and lyophilized before analysis.

After lyophilization of the preparation, the dried GBMs were resuspended at 10 mg/ml in Dubecco's PBS (D-PBS) containing 0.05% Tween-20. Three hundred to 500 μg of reconstituted GBMs (30-50 μl of resuspended GBMs brought to a total volume of 100 μl) were added in triplicate to wells of a HVPP 0.45 μm hydrophilic Durapore 96 well filtration plate pre-blocked with 3% non-fat dry milk. The wells were washed with D-PBS, and 200 μl of 1:2500 dilution of goat-anti-rabbit IgG conjugated with alkaline phosphatase in D-PBS were added. After 1 hr. incubation at 37°C, the plates were washed four times with D-PBS, and 200 μl of substrate (1 mg/ml PNPP in 10mM diethanolamine buffer with 0.5mM $MgCl_2$, pH 9.5, 0.1 mM levamisole). After 30 minutes the color reaction was stopped by adding 200 μl of 1 M NaOH after aspirating the substrate reaction mixture. The plates were read in a Dynatech Plate Reader at 410 nm. Results are expressed as OD units per well (per unit dry weight tissue).

Results

The results of IgG trapping in diabetic rats are depicted in Figure 3. In normal non-diabetic rats, IgG entrapment was slightly less than 0.75 μg/300 μg GBM. In contrast, untreated diabetic rats demonstrated a marked increase in IgG entrapment, with IgG levels approximately 2 times that found in the normal rats. Diabetic rats receiving aminoguanidine only had IgG levels in the GBM only slightly greater than that for the normal untreated rats, while diabetic rats receiving FFI-HA alone showed IgG levels as high as those for diabetic rat receiving no treatment at all. Finally, diabetic rats undergoing combination treatment, which includes aminoguanidine and FFI-HA, had IgG levels in the GBM that were lower than observed for untreated diabetic rats, or diabetic rats treated with FFI alone.

Discussion

The results described above, taken in conjunction with those of the prior examples, clearly supports a rationale for treating subjects exhibiting increased levels of AGEs in the tissues by administering aminoguanidine with FFI-HA. Although the administration of aminoguanidine alone in the present Example results in marked reduction of protein entrapment in the kidney, the administration of aminoguanidine alone in previous Examples does not substantially reduce the AGE levels in the particular tissues of study, such as in aorta *supra*, since the mechanism is only to prevent cross-linking of the glucosyl moieties to proteins rather than to potentiate the removal of such compounds. In effect, administration of FFI-HA solves this problem by inducing the uptake of AGEs by macrophages, thereby removing AGEs from tissue, and eventually resulting in delivery of the compounds to the kidneys for excretion. The underlying problem is that at the kidney these AGEs have the opportunity to reestablish cross-links to glomerular basement membrane proteins, which can trap plasma proteins that circulate through the kidney vasculature, like the immunoglobulins. Therefore, the efficacy of such combination therapy for individuals susceptible to increased levels of AGEs is clearer when the Examples are examined together.

EXAMPLE 4: AMINOGUANIDINE AND FFI-HA IN COMBINATION RESULTS IN INCREASED AGE EXCRETION FROM DIABETIC SUBJECTS

In the present Example, the levels of AGEs in urine of normal and diabetic rats were sampled to determine the ability of the drug combination of the invention to remove AGEs from the tissues and subsequently from the body of the subject. This Example is presented to further support the results of the IgG trapping assay described in Example 3, *supra*, which indicates indirectly that levels of AGEs present in the kidney cause pathology resulting in the trapping of normal healthy protein in the kidney vasculature.

Materials and Methods

To prepare urine from subjects for detection of AGE levels, urine was first collected over a 24 hour period and 1 ml was "cleared" by a short spin in the microfuge (3000 r.p.m. for 2 min.). Next, a 1:5 dilution of cleared urine was made with 0.3 M KH_2PO_4 (pH 7.4). This diluted volume was then passed through a Microcon filter unit system (10 kD molecular weight cut-off as per manufacturer's instructions, Amicon)to eliminate proteins of > 10,000 KD, thus allowing measurement of small proteins and peptides generated as a result of macrophage degradation of large AGE proteins. The low molecular weight fraction was resuspended to its original unfractionated volume, diluted 1:5 and 50 μl was added to each well in triplicate for determination of AGE content in an ELISA assay as described *supra*.

Results

Figure 4 shows the amounts of urinary AGE peptides produced in a 24 hour period for non-diabetic rats and for diabetic rat controls and treatment groups. According to the graph, the level of AGEs in urine of healthy rats is less than 4 U per 24 hour volume, while the untreated diabetic rats show relatively higher excreted levels of AGE peptides. Administration of aminoguanidine shows no effect on these levels, while administration of FFI-HA or both drugs increases the urinary output of AGE peptides. The greatest increase in AGE excretion is seen in rat urine samples of FFI-HA treated diabetic rats, while FFI-HA/aminoguanidine treated diabetic rat urine has only slightly lower levels of AGEs.

Discussion

The significance of the present Example is the great increase in urine output of AGEs in diabetic subjects undergoing combination therapy using aminoguanidine and FFI-HA. In comparison to normals, diabetic subjects demonstrated significantly greater excretion levels of AGEs. These levels increased after treatment with FFI-HA, both alone or in combination with AG. The dramatic increase in urine output of AGEs in diabetic subjects treated with FFI-HA or the combination therapy of the invention suggests that there is a corresponding decrease in AGE levels of the tissues.

EXAMPLE 5: EVALUATION OF HEMOGLOBIN-AGE LEVELS WITH COMBINATION TREATMENT

The levels of hemoglobin AGE were evaluated in diabetic rats treated with aminoguanidine, FFI, or aminoguanidine and FFI, and compared with normal and diabetic controls. The rats used were as described for Example 1, with the exception that rats were diabetic for 10 weeks prior to initiation of therapy with aminoguanidine (AG) and FFI-HA, and the rats were treated for a total of 10 weeks. The treatment protocol is summarized in Table 2.

TABLE 2

Treatment Protocol

ANIMAL GROUP	STZ 65 mg/kg I.V. Day 0	AG mg/kg Oral Given Daily	FFI-HA mg/kg I.P. Given Daily
Normal	-	-	-
Diabetic	+	-	-
Diabetic	+	40	-
Diabetic	+	125	-
Diabetic	+	400	-
Diabetic	+	-	10
Diabetic	+	40	10
Diabetic	+	125	10
Diabetic	+	400	10

The results of this experiment are shown in Figure 5. The data show a dramatic effect in reducing the level of hemoglobin-AGE with combination therapy of aminoguanidine (125 and 400 mg/kg/day) and FFI-HA, compared to aminoguanidine or FFI alone, and to untreated diabetic controls.

In this study, the lack of effectiveness of lower doses of AG (40 mg/kg/day) may be explained by the fact that not enough drug permeates the red cell. However, combination therapy was highly efficacious at the two higher doses tested.

EXAMPLE 6: CYTOKINE MODULATION OF THE RECEPTOR FOR ADVANCED GLYCOSYLATION END-PRODUCTS ON MACROPHAGES *IN VITRO*

Materials and Methods

Radioligand Binding Assay. RAW 264.7 cells were maintained in RPMI-1640 medium containing 10% fetal calf serum. Cells were contacted with MIF, MIP-1ß, and GM-CSF at the concentrations given in the corresponding Figures (6, 7, and 8) and incubated one or three days. The cells were washed three times with cold RPMI-1640 without fetal calf

serum prior to use. The appropriate mixture of iodinated 12 week AGE-BSA and cold competitor ligand was made in serum free RMPI-1640 just prior to assay. One ml of the ligand mixture was added to the appropriate wells and the plates are placed at 4°C on a rocking platform for 4 hours. The mixture was removed and the cells were washed 5 times with cold serum free RPMI-1640. To each well were added 1.0 ml of 1.0 N NaOH for 15 to 30 minutes. The disrupted cells were removed using a cotton tipped swab. After placing the swab in the appropriate tube for gamma counting, the medium remaining in each well was collected and added to the tubes containing the swabs. Each well was washed twice with 0.5 ml of 1.0 N NaOH, which was added to the appropriate tubes. The samples were counted in an LKB gamma counter and the final numbers were used to determine the % specific binding.

Results

When macrophages are incubated *in vitro* with inflammatory cytokines or chemokines or colony stimulating factors, the effects include an increase in AGE binding. Following incubation of cells with MIF (Figure 7), MIP-1β (Figure 8), and GM-CSF(Figure 9), AGE-receptor-mediated binding increased to a degree similar to that observed with FFI-HA alone. However, in this experiment, treatment of macrophages *in vitro* with MIP-1α, IL-6, or IL-1 showed no effect on binding of AGEs to macrophage (data not shown). As anticipated, treatment with the immunosuppressive cytokine IL-10 resulted in a 20% reduction in AGE binding (data not shown). Taken together, these studies suggest that modulation of the receptor for AGEs on macrophages by FFI-HA may be mediated in part by effects on cytokine or chemokine induction. These data clearly indicate that certain cytokines, chemokines, and colony stimulating factors are effective to stimulate AGE binding and uptake by macrophages, and directly suggest effective combination therapy with GM-CSF, MIF, MIP-1β, or other similar inflammatory cytokines, chemokines, or colony stimulating factors and AGE inhibits, such as aminoguanidine or an AGE reversing agent, *in vivo*.

This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the

invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

Various references are cited throughout this specification, each of which is incorporated herein by reference in its entirety.

WHAT IS CLAIMED IS:

1. A composition for promoting the sequestration and removal from the body of an animal of macromolecules that have undergone advanced glycosylation comprising an admixture of an agent capable of causing the body to increase its activity of recognizing and removing the macromolecules, and a compound capable of inhibiting the formation of advanced glycosylation endproducts by reacting with an active carbonyl moiety on an early glycosylation product and inhibits formation of AGE-mediated crosslinks.

2. The composition of claim 1 wherein the agent is selected from the group consisting of an advanced glycosylation endproduct, an advanced glycosylation endproduct bound to a carrier, a cytokine or chemokine that stimulates the body to increase the recognizing and removing activity toward the target macromolecules, a colony stimulating factor, and mixtures thereof.

3. The composition of claim 1 wherein the agent is selected from the group consisting of 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole, the hexanoic acid derivative thereof, and 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole bound to a carrier.

4. The composition of claim 2 wherein the carrier is selected from the group consisting of a carbohydrate, a protein, a lipid, a synthetic polypeptide, a biocompatible natural or synthetic resin, and mixtures thereof.

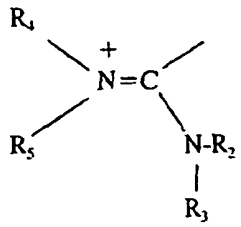
5. The composition of claim 1, further comprising a co-stimulatory agent.

6. The composition of claim 1, wherein the compound is selected from the group consisting of aminoguanidine, α-hydrazinohistidine, lysine, an analog of aminoguanidine, and mixtures thereof.

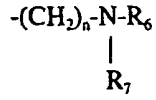
7. The composition of claim 6, wherein the analog is selected from the group consisting of hydrazine derivatives of the formula:

$$R - \underset{\displaystyle R_1}{N}\text{-}NH_2$$

wherein R is a group of the formula



and R_1 is hydrogen or a lower alkyl group of 1-6 carbon atoms, a hydroxyethyl group, or together with R_2 may be a lower alkylene bridge of 2-4 carbon atoms; R_2 is hydrogen or a lower group alkyl of 1-6 carbon atoms or together with R_1 or R_3 is a lower alkylene bridge of 2-4 carbon atoms, amino, hydroxy, or an aminoalkylene group of the formula



wherein n is an integer of 2-7 and R_6 and R_7 are independently a lower alkyl group of 1-6 carbon atoms or together form a part of a cycloalkyl or heterocyclic ring containing from 1 to 2 heteroatoms, of which at least one is nitrogen; and the second of the heteroatoms is selected from the group consisting of nitrogen, oxygen, and sulfur; with the proviso that when the second of the heteroatoms of the heterocyclic ring is nitrogen and forms a piperazine ring, it may be optionally substituted by a substituent that is identical to the portion of the compound on the first nitrogen of the piperazine ring; R_3 is hydrogen, a lower alkyl group of 1-6 carbon atoms, or together with R_2 or R_4 is a lower alkylene bridge of 2-4 carbon atoms; R_4 is hydrogen, a lower alkyl group of 1-6 carbon atoms or together with R_3 is a lower alkylene bridge of 2-4 carbon atoms; or an amino group; R_5 is hydrogen, or a lower alkyl group of 1-6 carbon atoms; with the proviso that at least one of R_1, R_2, R_3, R_4 or R_5 is other than hydrogen; or R is an acyl or a lower alkylsulfonyl group of up to 10 carbon atoms and R_1 is hydrogen; and their pharmaceutically acceptable acid addition salts.

8. The composition of claim 1 wherein the agent capable of causing the body to increase its activity of recognizing and removing the macromolecules is covalently coupled to the compound capable of inhibiting the formation of advanced glycosylation endproducts.

9. The composition of claim 1 wherein the agent is selected from the group consisting of FFI, and the compound is aminoguanidine.

10. A pharmaceutical composition for administration to an animal to promote the sequestration and removal from the animal's body of a macromolecule that has undergone advanced glycosylation, comprising a therapeutically effective amount of the composition of claim 1, and a pharmaceutically acceptable carrier.

11. A method for averting the adverse sequelae of the accumulation of advanced glycosylation endproducts in the body of an animal, comprising introducing into the body a therapeutically effective amount of an agent capable of causing the body to increase its activity of recognizing and removing macromolecules that have undergone advanced glycosylation, and a therapeutically effective amount of a compound capable of inhibiting the formation of advanced glycosylation endproducts by reacting with an active carbonyl moiety on an early glycosylation product and inhibits formation of AGE-mediated crosslinks.

12. The method of claim 11 wherein the agent is selected from the group consisting of an advanced glycosylation endproduct, an advanced glycosylation endproduct bound to a carrier, a cytokine or chemokine that stimulates the phagocytic cells in the body to increase the recognizing and removing activity toward the macromolecules, a colony stimulating factor, and mixtures thereof.

13. The method of claim 11 wherein the agent is selected from the group consisting of 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole, the hexanoic acid derivative thereof, and 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole bound to a carrier.

14. The method of claim 13 wherein the carrier is selected from the group consisting of a carbohydrate, a protein, a lipid, a synthetic polypeptide, a biocompatible natural or synthetic resin, and mixtures thereof.

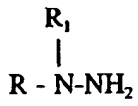
15. The method of claim 11 further comprising administration of a co-stimulatory agent.

16. The method of claim 11 wherein the agent is administered parenterally.

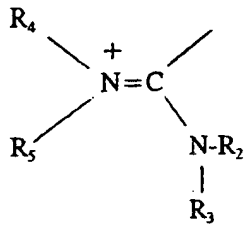
17. The method of claim 11, herein the agent is administered orally.

18. The method of claim 11, wherein the compound is selected from the group consisting of aminoguanidine, α-hydrazinohistidine, lysine, an analog of aminoguanidine, and mixtures thereof.

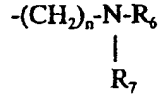
19. The method of claim 17, wherein the analog is selected from the group consisting of hydrazine derivatives of the formula:



wherein R is a group of the formula



and R_1 is hydrogen or a lower alkyl group of 1-6 carbon atoms, a hydroxyethyl group, or together with R_2 may be a lower alkylene bridge of 2-4 carbon atoms; R_2 is hydrogen or a lower group alkyl of 1-6 carbon atoms or together with R_1 or R_3 is a lower alkylene bridge of 2-4 carbon atoms, amino, hydroxy, or an aminoalkylene group of the formula



wherein n is an integer of 2-7 and R_6 and R_7 are independently a lower alkyl group of 1-6 carbon atoms or together form a part of a cycloalkyl or heterocyclic ring containing from 1 to 2 heteroatoms, of which at least one is nitrogen; and the second of the heteroatoms is selected from the group consisting of nitrogen, oxygen, and sulfur; with the proviso that when the second of the heteroatoms of the heterocyclic ring is nitrogen and forms a piperazine ring. it may be optionally substituted by a substituent that is identical to the portion of the compound on the first nitrogen of the piperazine ring; R_3 is hydrogen, a lower alkyl group of 1-6 carbon atoms, or together with R_2 or R_4 is a lower alkylene

bridge of 2-4 carbon atoms; R_4 is hydrogen, a lower alkyl group of 1-6 carbon atoms or together with R_3 is a lower alkylene bridge of 2-4 carbon atoms; or an amino group; R_5 is hydrogen, or a lower alkyl group of 1-6 carbon atoms; with the proviso that at least one of R_1, R_2, R_3, R_4 or R_5 is other than hydrogen; or R is an acyl or a lower alkylsulfonyl group of up to 10 carbon atoms and R_1 is hydrogen; and their pharmaceutically acceptable acid addition salts.

20. The method of claim 11 wherein the agent capable of causing the body to increase its activity of recognizing and removing the macromolecules is covalently coupled to the compound capable of inhibiting the formation of advanced glycosylation endproducts.

21. The method of claim 20 wherein the agent is FFI and the compound is aminoguanidine.

22. The method of claim 11 wherein the agent and the compound are administered simultaneously; the agent is administered in a bolus and the compound is administered by continuous infusion within 24 hours of each other; or the agent is administered parenterally and the compound is administered orally within 24 hours of each other.

FIG.1

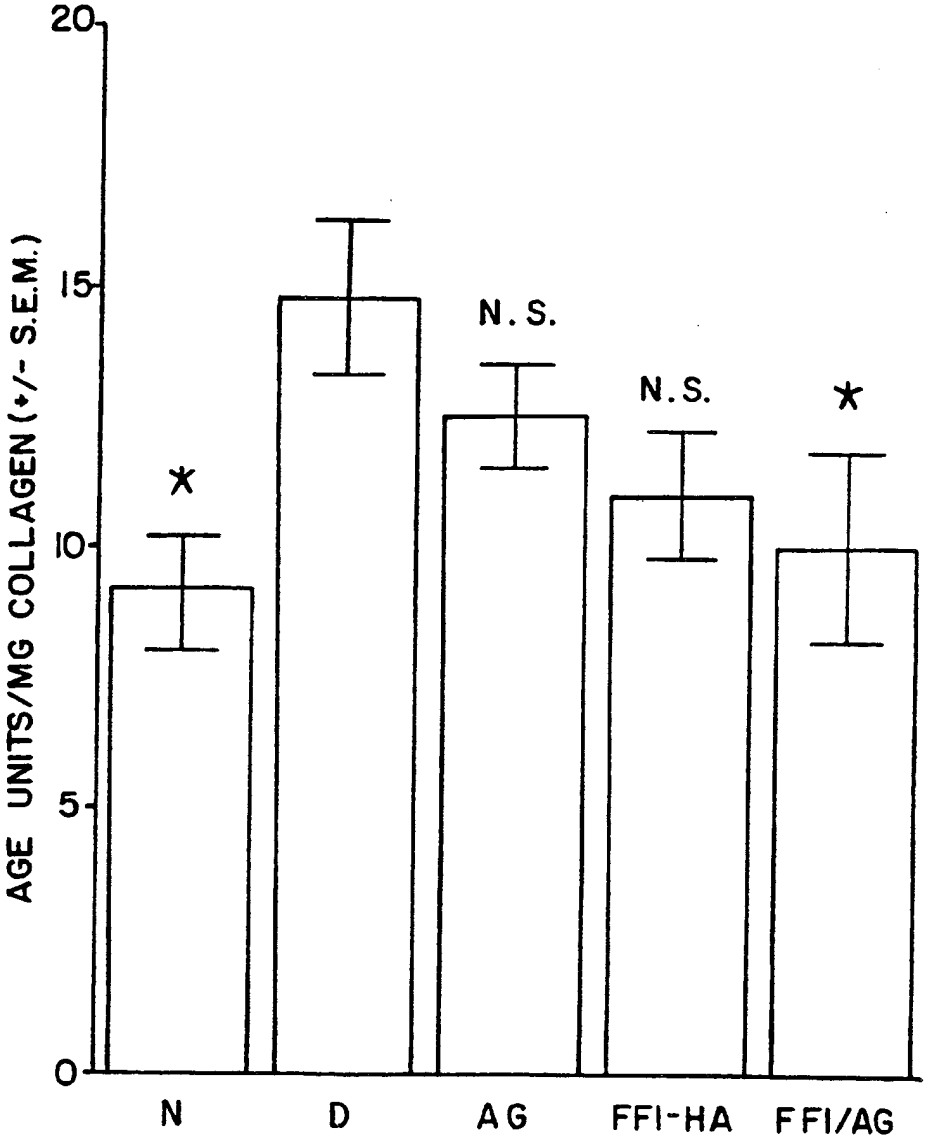
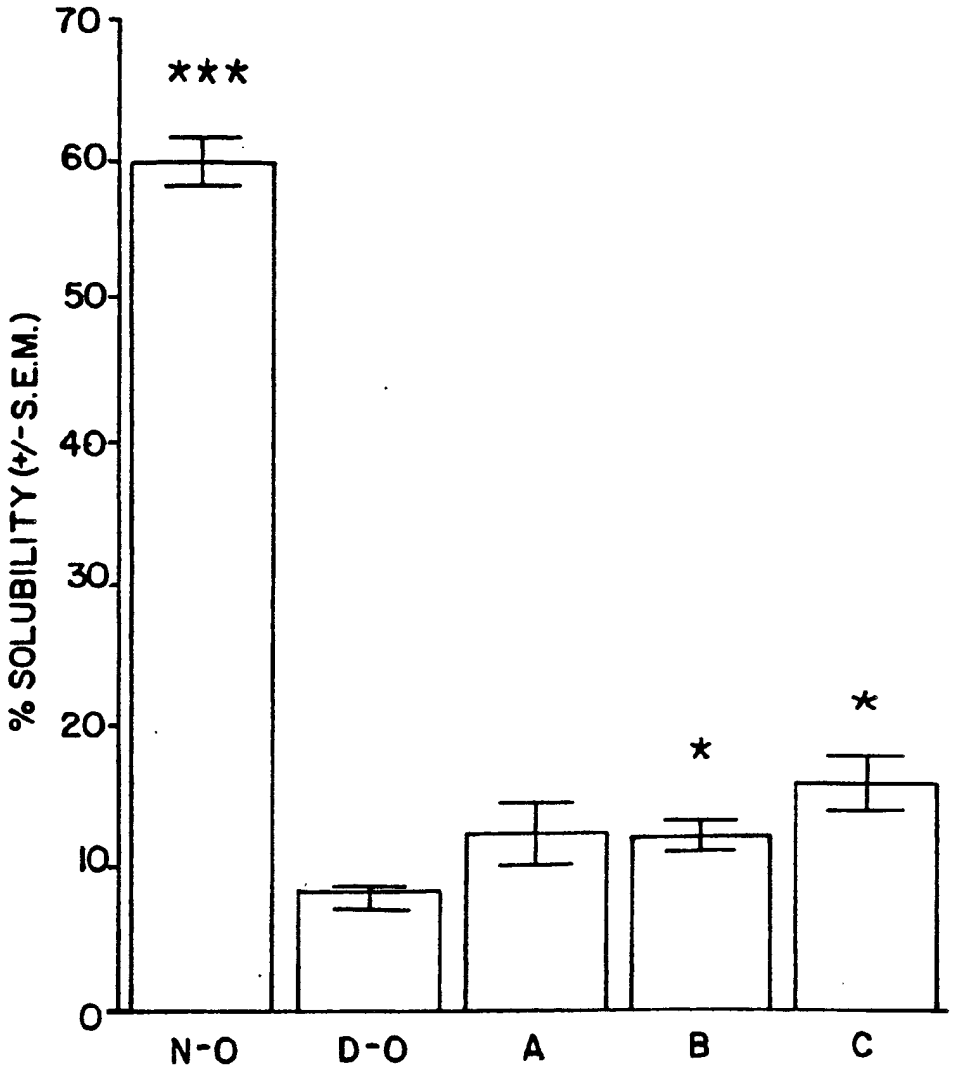


FIG.2



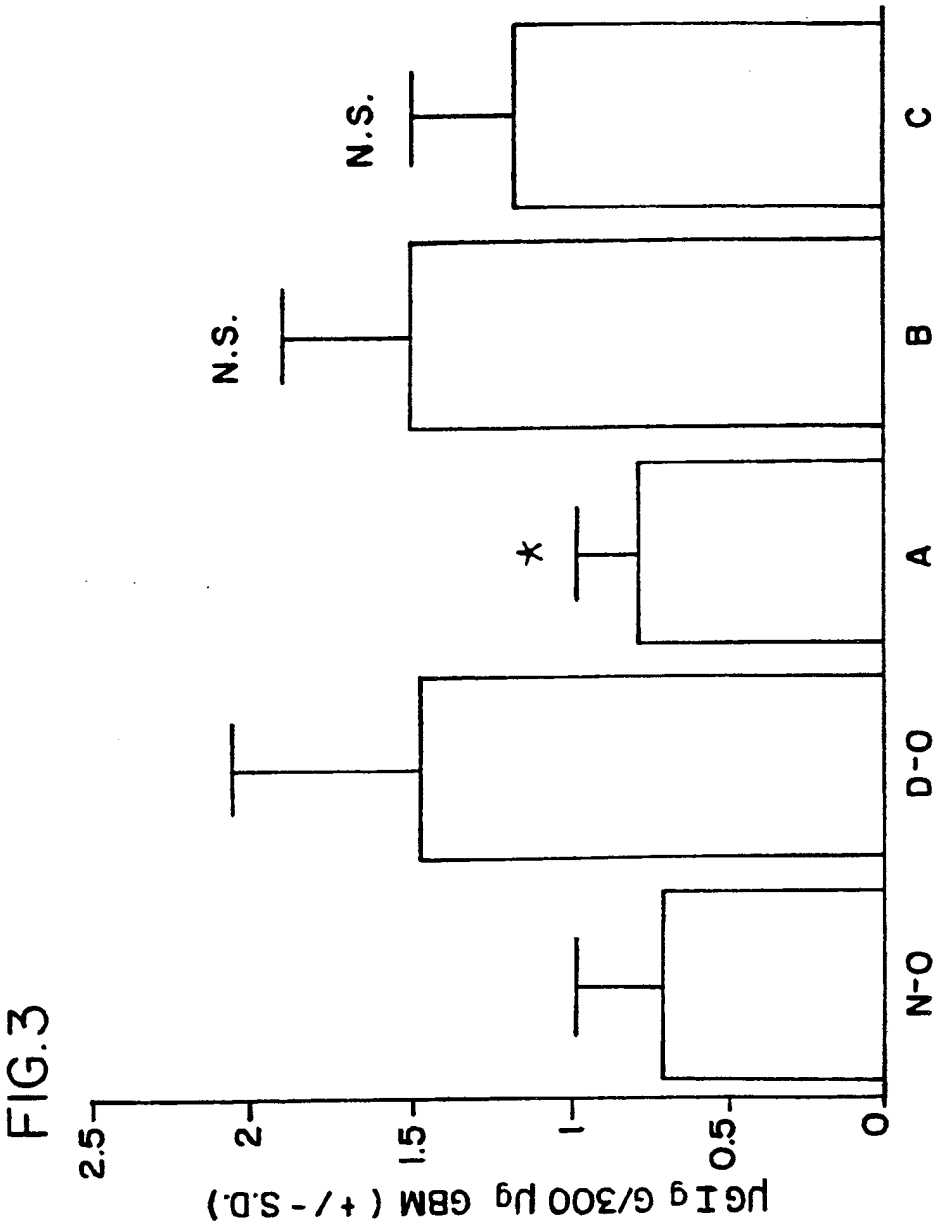
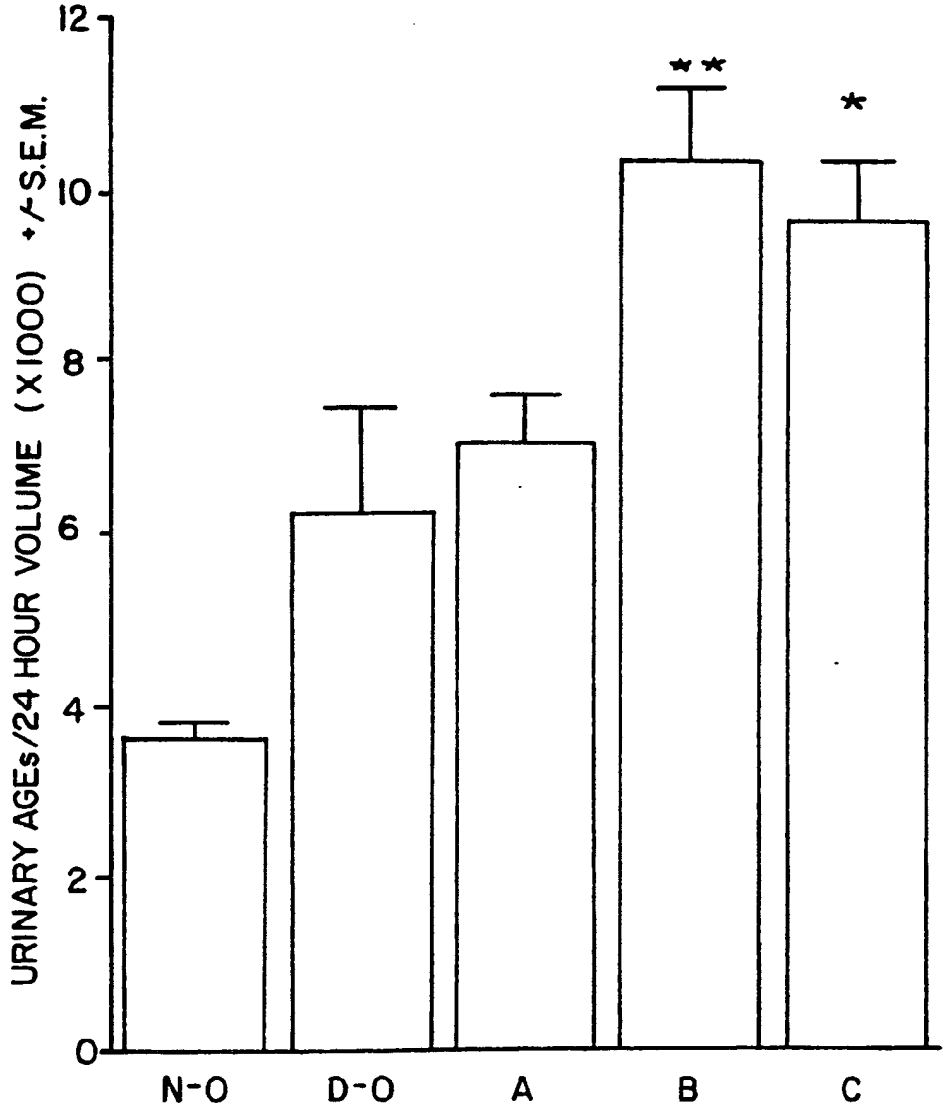


FIG.3

FIG. 4



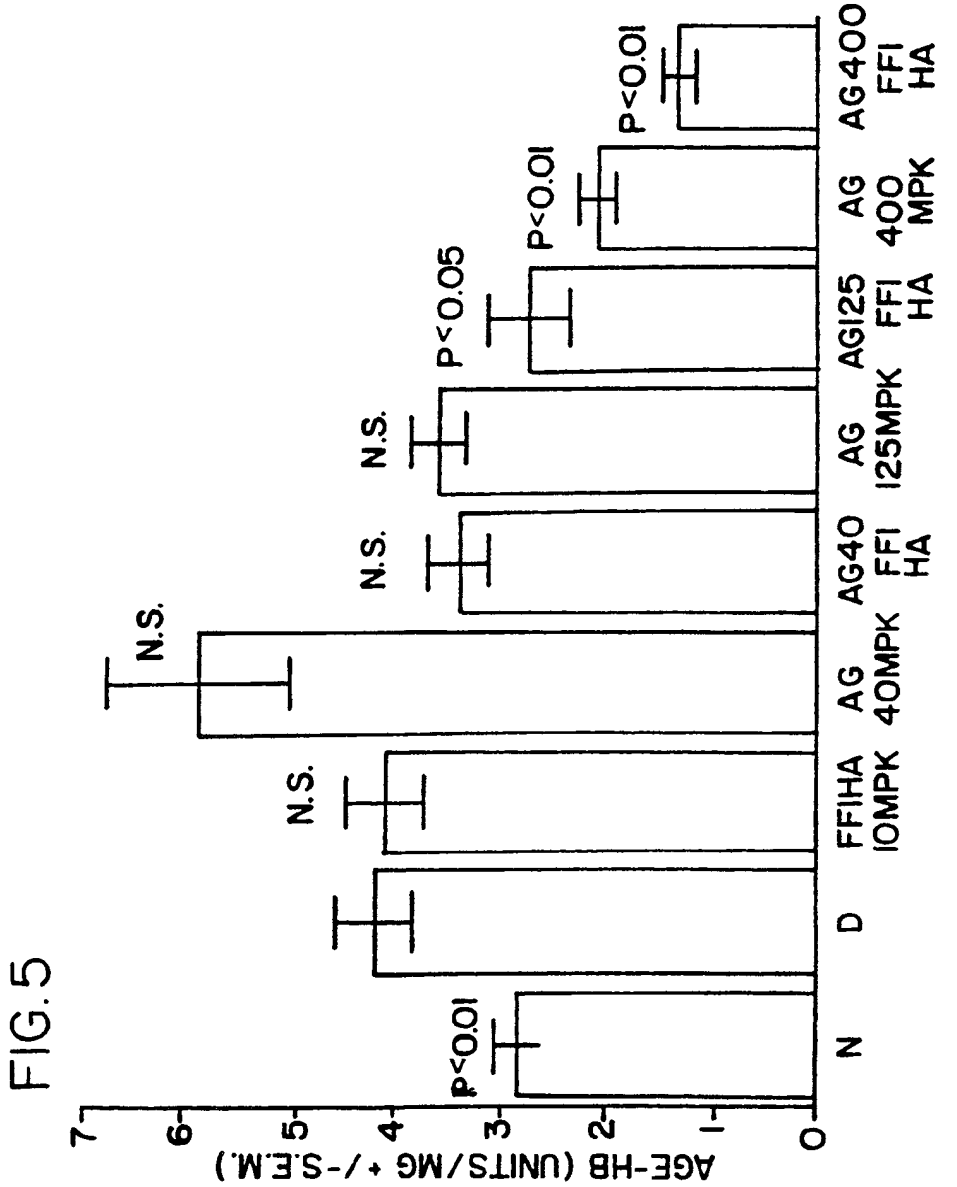

AGE-HB (UNITS/MG +/-S.E.M.)
0
1
2
3
4
5
6
7
P<0.01
N.S.
N.S.
N.S.
P<0.05
P<0.01
P<0.01
N
D
FFIHA 10MPK
AG 40MPK
AG40 FFI HA
AG 125MPK
AG125 FFI HA
AG 400 MPK
AG400 FFI HA

FIG.5

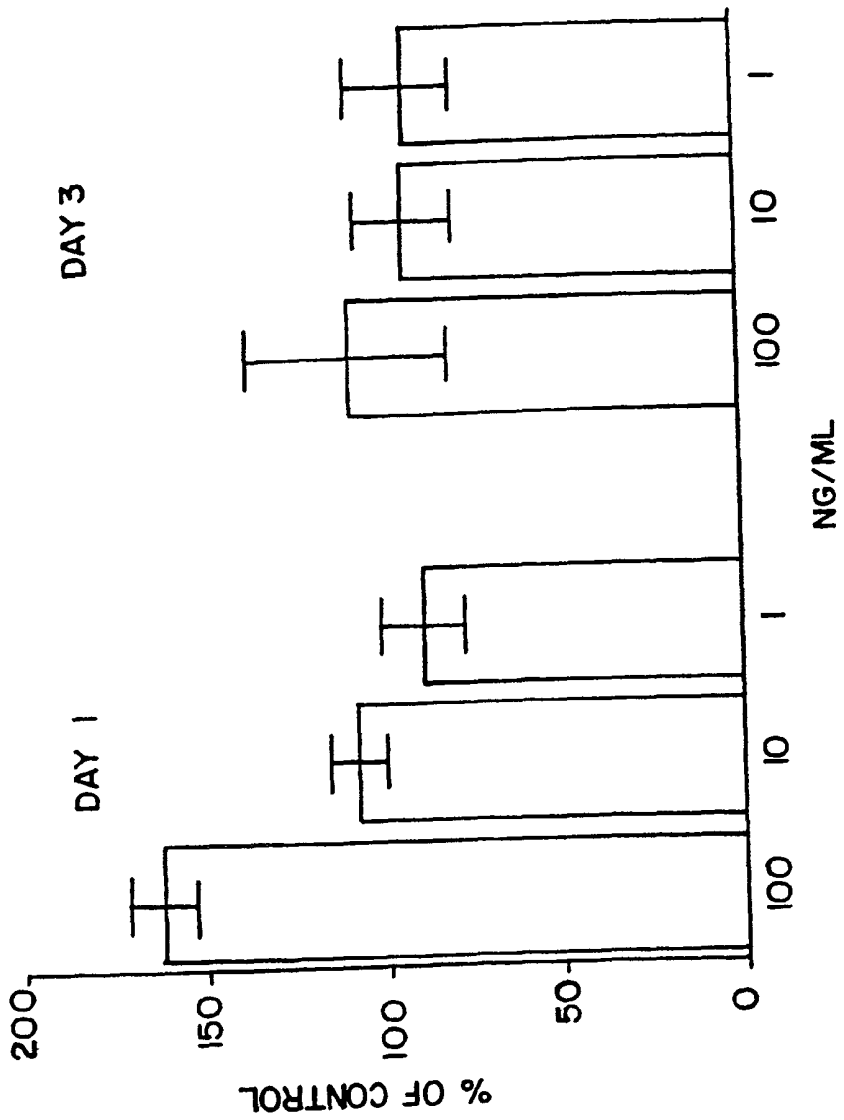
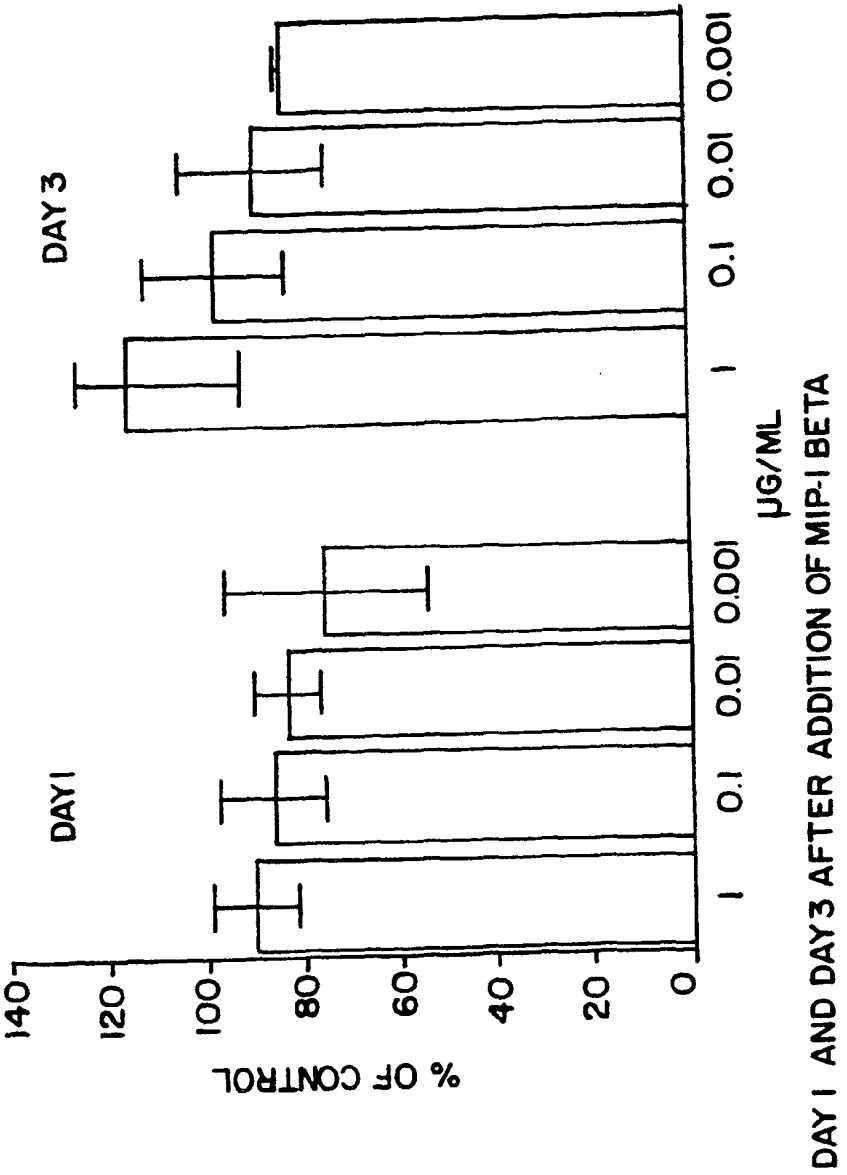


FIG.6

FIG. 7


DAY 1 AND DAY 3 AFTER ADDITION OF MIP-1 BETA
% OF CONTROL
0
20
40
60
80
100
120
140
DAY 1
DAY 3
1
0.1
0.01
0.001
1
0.1
0.01
0.001
μG/ML

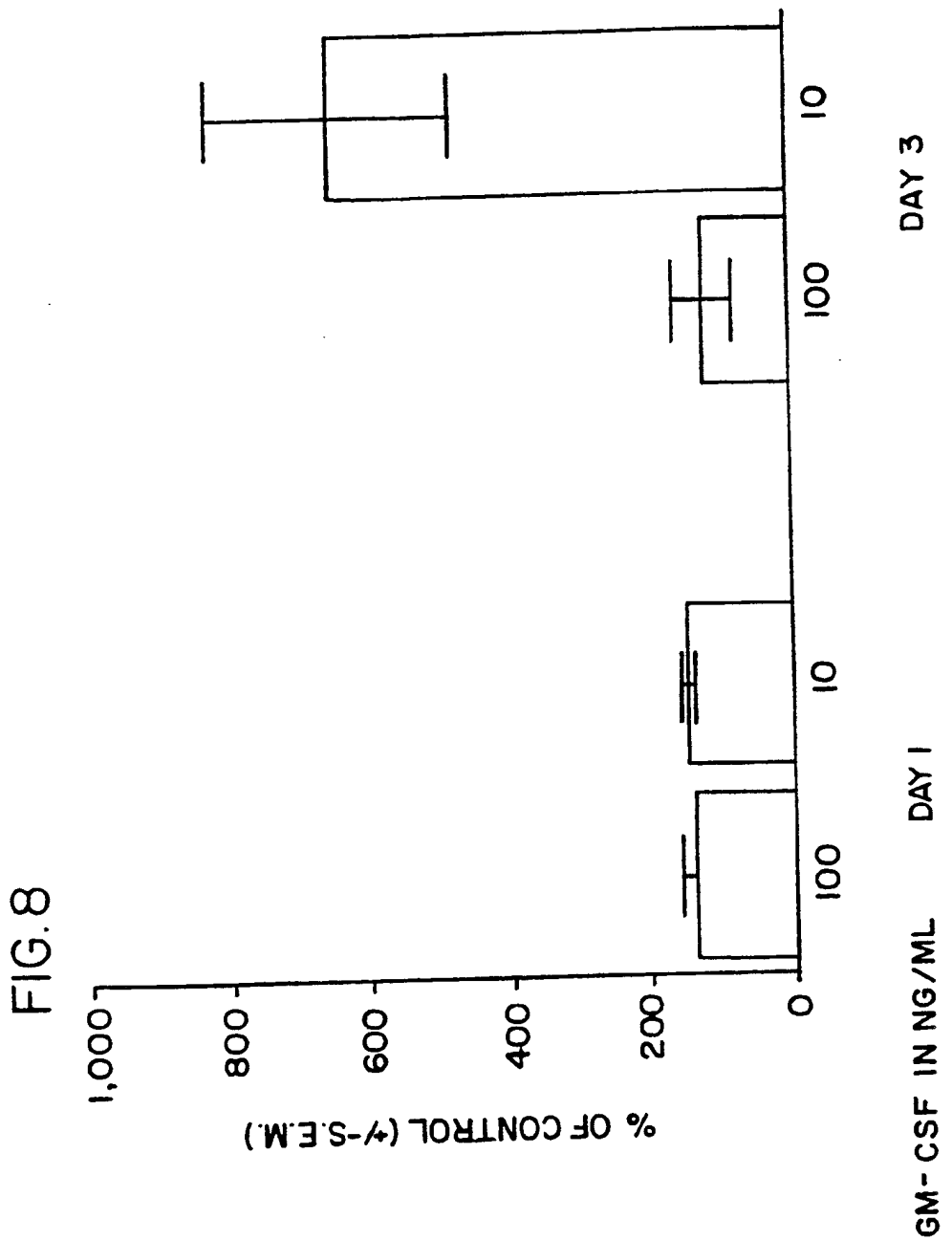

% OF CONTROL (+-S.E.M.)
1,000
800
600
400
200
0
100
10
100
10
DAY 1
GM-CSF IN NG/ML
DAY 3

FIG. 8

INTERNATIONAL SEARCH REPORT

Ir tional Application No
PCT/US 96/13258

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/38 A61K31/415

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BLOOD PURIFICATION, 13 (3-4). 1995. 160-170., XP000614744 BUCALA R ET AL: "Advanced glycosylation endproducts in diabetic renal disease: Clinical measurement, pathophysiological significance, and prospects for pharmacological inhibition" see abstract ---	1-22
A	US,A,4 900 747 (VLASSARA HELEN ET AL) 13 February 1990 cited in the application see abstract -----	1-22

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 1997	05.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Leherte, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/13258

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 11-22 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

In view of the large number of compounds which are defined by the wording of the claims, the search has been performed on the general idea and compounds mentioned in the examples of the description.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos..

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/US 96/13258

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-4900747	13-02-90	US-A-	4665192	12-05-87
		CA-A-	1323565	26-10-93
		US-A-	5316754	31-05-94
		US-A-	5585344	17-12-96
		US-A-	5202424	13-04-93
		AU-B-	583034	20-04-89
		AU-A-	4153185	11-10-85
		CA-A-	1250587	28-02-89
		CA-A-	1294218	14-01-92
		DE-D-	3587667	05-01-94
		DE-T-	3587667	17-03-94
		EP-A-	0175764	02-04-86
		EP-A-	0322402	28-06-89
		JP-A-	5172813	13-07-93
		JP-T-	61501706	14-08-86
		WO-A-	8504169	26-09-85
		US-A-	5534540	09-07-96
		US-A-	5476849	19-12-95
		US-A-	5468777	21-11-95
		US-A-	5514676	07-05-96
		US-A-	5128360	07-07-92
		US-A-	5175192	29-12-92
		US-A-	5128122	07-07-92
		US-A-	5140048	18-08-92
		US-A-	5096703	17-03-92
		US-A-	5106877	21-04-92
		US-A-	5114943	19-05-92
		US-A-	5258381	02-11-93
		US-A-	5218001	08-06-93
		US-A-	5130324	14-07-92
		US-A-	5130337	14-07-92
		US-A-	4758583	19-07-88
		US-A-	5238963	24-08-93
		US-A-	5254593	19-10-93
		US-A-	5221683	22-06-93
		US-A-	5334617	02-08-94
		US-A-	5288615	22-02-94
		US-A-	5356895	18-10-94
		US-A-	5243071	07-09-93
		US-A-	5262152	16-11-93

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/US 96/13258

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4900747		US-A- 5272176	21-12-93
		US-A- 5399560	21-03-95
		US-A- 5318982	07-06-94
		US-A- 5358960	25-10-94
		US-A- 5326779	05-07-94
		US-A- 5272165	21-12-93
		AU-B- 600306	09-08-90
		AU-A- 7827587	17-03-88
		EP-A- 0259893	16-03-88
		JP-A- 63146833	18-06-88